SEROLOGICAL DIFFERENTIATION OF STERIC ISOMERS (ANTIGENS CONTAINING TARTARIC ACIDS)

Second Paper

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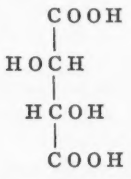
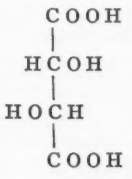
In experiments reported already (1) it has been demonstrated that the presence of optically isomeric groups in two otherwise identical antigens suffices to bring about a difference in their serological properties. The case examined was that of the levo- and dextro-phenyl (paraaminobenzoylamino) acetic acids which after diazotization were combined with proteins. The resulting antigens were distinctly different when tested with the corresponding precipitating immune sera. Considering that steric isomerism presumably is of significance for the specificity of natural antigens, it seemed desirable to extend the studies to other instances and also to proceed to the examination of compounds containing more than one asymmetric carbon atom.

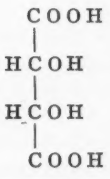
For this purpose the tartaric acids were chosen which, as is well known, contain two asymmetric carbon atoms and which exist in three isomeric forms, namely, levo-, dextro-, and mesotartaric acid, aside from the racemic mixture of *l*- and *d*-acid. The following formulae represent the three forms



levotartaric acid



dextrotartaric acid



mesotartaric acid

A method for attaching these substances to protein was found in

preparing first a compound with paraphenylenediamine in which one of the amino groups is linked to one of the carboxyls of the tartaric acid according to the following formula: $NH_2C_6H_4NHCO\,(CHOH)_2\,COOH$. This substance was diazotized and coupled to protein. After a few unsuccessful attempts to prepare this acyl derivative directly by condensation of tartaric acid with paraphenylenediamine, it was found possible to combine tartaric acid with paranitraniline and to reduce the resulting substance to the amino compound.

EXPERIMENTAL

Levo- and mesotartaric acids were prepared according to the methods of Holleman (2) and Marckwald (3). The meso acid was obtained in the form of its calcium salt which forms characteristic square crystals easily distinguishable from those of the racemic mixture. From this calcium salt the free acid was isolated by adding less than the calculated amount of a dilute solution of sulfuric acid and allowing the material to stand at room temperature for 1 day. The filtered solution was concentrated to a small volume at 40° by vacuum distillation. The mesotartaric acid crystallized on cooling and was dried *in vacuo* at 45° (M.Pt. after two recrystallizations 142–143°C.)

Frankland and Slator (4) prepared a ditoluidide of tartaric acid $(C_7H_7NHCO)_2(CHOH)_2$ by heating *p*-toluidine (2 mols) and tartaric acid (1 mol) for 10 hours in an oil bath at 180–185°. They established by subsequent hydrolysis of the substance that the tartaric acid had not undergone any appreciable racemization during the process.

Applying a similar method to paranitraniline and using instead of 2 mols only 1 mol of paranitraniline for 1 mol of tartaric acid we found that besides a non-acid product (probably the dinitranilide of tartaric acid) paranitrotartranilic acid ($NO_2C_6H_4NHCO(CHOH)_2COOH$) in good yield was also obtained. Accordingly the derivatives of levo-, dextro-, and mesotartaric acid were prepared by the following process:

15 gm. of tartaric acid were ground with 13.8 gm. of paranitraniline and heated (in a test tube) in a paraffin bath at 170°C. When, after about 5 minutes, bubbles began to form in the molten mass the temperature was lowered to 155–160°. In the case of the levo- and dextro-tartaric acid but not in that of the meso-acid the fluid began to thicken after about 15 minutes and later solidified. After heating for about 40 minutes in all, the mass was broken up by heating with water and a little alcohol, the volume was brought to 600 cc. with water and a solution of NaOH was added until the liquid reacted neutral to litmus. The insoluble material was removed by filtration and the solution was concentrated on the steam bath to a volume of 200 cc. A little insoluble material which separated was filtered off after cooling to room temperature and enough 10 per cent hydrochloric acid was added to make the solution acid to congo red. The paranitrotartranilic acid pre-

cipitated as a light yellow powder which was filtered off, washed with water and dried *in vacuo*. The yield was approximately 12 gm. of the levo- and dextro-compounds and somewhat less of the meso-compound. Recrystallized from water the levo- and dextro acids form fine yellow needles. The meso acid crystallizes in pale yellow microscopic platelets.

Levo-paranitrotartranilic acid $C_{10}H_{10}O_7N_2$. M.Pt. 211–212°.

Analysis: calculated: C 44.44 H 3.70
found: " 44.76 " 3.40

270 mg. neutralized 9.95 cc. 1/10 N NaOH; calculated: 10 cc. A 1% solution in methyl alcohol gave at 22° a rotation of −2.40 in a 2 dm. tube with sodium light, $[\alpha]_D$ −120.

Dextro-paranitrotartranilic acid M.Pt. 212–213°.

270 mg. neutralized 9.93 cc. 1/10 N NaOH; calculated: 10 cc. A 1% solution in methyl alcohol gave at 22° a rotation of +2.40 in a 2 dm. tube, $[\alpha]_D$ +120.

Meso-paranitrotartranilic acid M.Pt. 193–194°.

270 mg. neutralized 9.95 cc. 1/10 N NaOH; calculated 10 cc. A 1% solution in methyl alcohol showed no rotation.

Reduction of Nitrotartranilic Acid to the Amino Compound.—20 gm. of crude finely ground para-nitrotartranilic acid were suspended in 1.5 liters of ⅓ normal hydrochloric acid. About 36 gm. of zinc dust were added in small portions over a period of 15 minutes with constant stirring. The paranitrotartranilic acid disappeared gradually within ½ hour. The excess of zinc was filtered off and to the solution which showed faint acidity to congo red, 70 gm. of sodium acetate were added and hydrogen sulfide was passed through until the zinc had been completely precipitated. After filtration the solution was neutralized with sodium hydroxide. In the case of the meso-paraaminotartranilic acid it was neutralized before filtering off the zinc sulfide to avoid a loss of substance by precipitation in the acid solution. The solution was concentrated at 40° by vacuum distillation to a volume of 200 cc. and after removing a trace of insoluble material it was cooled in a freezing mixture and enough concentrated hydrochloric acid was added to make it weakly acid to congo. The paraaminotartranilic acid came out of solution and was filtered off. It was washed with water, alcohol and ether and dried *in vacuo*. The yield was approximately 10 gm. By recrystallization of the levo- and dextro-compounds from 25 parts of water with the addition of some decolorizing carbon they were obtained in the form of microscopic white needles or platelets.

The meso-compound which was almost white was purified by dissolving in water and the required amount of sodium hydroxide, and reprecipitation with acid; white microscopic rectangular platelets after recrystallization from water.

Analyses:

Levo-paraaminotartranilic acid $C_{10}H_{12}O_5N_2$.

240 mg. neutralized 9.85 cc. 1/10 N NaOH; calculated 10 cc.

Kjehldahl nitrogen analysis: Found N. 11.76%; calculated 11.67%.

A water solution containing 480 mg. of the substance and 2.6 cc. of normal HCl in a volume of 15 cc. gave at 25°C. a rotation of −6.31 in a 2 dm. tube; $[\alpha]_D$ −98.6.

Dextro-paraaminotartranilic acid $C_{10}H_{12}O_5N_2$

240 mg. neutralized 9.90 cc. 1/10 N NaOH; calculated 10 cc.

Kjehldahl nitrogen analysis: Found N. 11.55%, calculated 11.67%.

A water solution containing 480 mg. of the substance and 2.6 cc. of normal HCl in a volume of 15 cc. gave at 25°C. a rotation of +6.35 in a 2 dm. tube; $[\alpha]_D$ +99.2.

Meso-paraaminotartranilic acid $C_{10}H_{12}O_5N_2$

240 mg. neutralized 9.90 cc. 1/10 N NaOH; calculated 10 cc.

Kjehldahl nitrogen analysis: Found N. 11.62%; calculated 11.67%.

The amino compounds became dark but did not melt when heated to 285°C.

The meso-paraaminotartranilic acid obtained must consist of a racemic mixture of two optically active compounds since the combination with paranitraniline destroys the symmetry of the molecule. This circumstance does not interfere at all with the conclusions to be drawn from the following experiments and consequently no attempt was made to resolve the product into its components.

The levo-, dextro-, and meso-paraaminotartranilic acids will be designated as *l*-, *d*-, and *m*-acid and likewise the azoproteins prepared from these amino acids and the immune sera obtained by immunization with the azoproteins will be referred to as *l*-, *d*-, and *m*-antigens, and *l*-, *d*-, and *m*-immune sera, respectively.

Preparation of the l-, d-, and m-Antigens for Immunization.—The *l*- and *d*-paraaminotartranilic acids were coupled to protein in the following manner:

7.6 gm. were dissolved in 200 cc. water and 85 cc. of normal HCl and diazotized with the required amount of sodium nitrite at a temperature of 0–5°C. with starch iodide paper as indicator. The diazo solution was diluted with ice water to a volume of 1200 cc.

800 cc. of this solution were added to a cold mixture of 500 cc. horse serum and 100 cc. normal sodium carbonate and the mixture, chilled with ice, was kept weakly alkaline to phenolphthalein by adding frequently small quantities of sodium carbonate solution. The coupling proceeded slowly (test with alkaline R salt solution for the presence of free diazo compound) and the diazo compound was used up after about 1½ hours. Then the remaining 400 cc. of diazo solution and 50 cc. of normal sodium carbonate were added (further addition of sodium carbonate and cooling as above). The coupling was finished after about 1 hour. Under the conditions described the meso compound coupled faster but for the sake of uniformity the operations were carried out in exactly the same manner as with the *l*- and *d*- substances.

By acidification with hydrochloric acid the azoprotein was precipitated and after filtration it was dissolved in a small volume of water by addition of a little normal sodium carbonate. It was reprecipitated from this solution with a large quantity of alcohol. The precipitated azoprotein was ground in a mortar to a thin paste, brought up to a volume of 950 cc. with water and the necessary amount of a salt solution to make the salt concentration approximately 1%. As a preservative 50 cc. of 5% phenol solution were added.

Immunization.—Three batches of six rabbits each were injected intraperitoneally at weekly intervals. Each rabbit received 12 cc. of the antigen per injection. Test bleedings were made 1 week after the third and the fourth injections. Three to four sera of sufficient strength were obtained in each lot after three or four injections.

Antigens for the Tests.—These were prepared in the same way as the antigens for immunization, chicken serum being used instead of horse serum. The azoproteins were precipitated with acid, washed with water and brought into solution by means of sodium carbonate. The quantity of antigen in the solution was determined by precipitation with alcohol and weighing the dried substance. The dilutions given in the tables are in terms of a 5% stock solution.

The intensity of the reaction is indicated as follows: o, f. tr. (faint trace), tr. (trace), ±, +, +±, etc.

The experiments presented in Tables I to III show an almost complete specificity of the *l*- and *d*- antigens. The *l*- and *d*- immune sera give rather weak group reactions with the *m*-antigen; the *m*-immune sera gave practically no group reactions.

Tests were also made with an antigen made from racemic tartaric acid. It was found that this substance, as was to be expected, reacts like a mixture of *l*- and *d*-antigens, *i.e.*, it is precipitated by both the *l*- and *d*-immune sera like the homologous antigens only somewhat weaker according to the lower concentration of the respective homologous antigens. With the *m*-immune sera there were only faint reactions similar to those of the *l*- and *d*-antigens. These results with the racemic preparation are not tabulated.

Other immune sera, namely, two *l*-, three *d*- and three *m*- sera gave results entirely in agreement with those presented in the tables.

In the following experiments presented in Tables IV,*a*, IV,*b*, and IV,*c*, the inhibiting effect on the precipitin reaction of the tartaric acids, the paraaminotartranilic acids and some other substances used for

TABLE I, *a*

To 0.2 cc. of the diluted antigens (prepared with chicken serum) were added 2 capillary drops of *l*-immune serum.

Readings taken after:	*l*-Antigen Dilution 1:				*d*-Antigen Dilution 1:				*m*-Antigen Dilution 1:			
	20	100	500	2500	20	100	500	2500	20	100	500	2500
1 hr. at room temperature	±	+	+	tr.	0	0	0	0	0	tr.	0	0
3 hrs. at room temperature	+	+±	+±	tr.	0	0	0	0	f. tr.	tr.	f. tr.	0
Night in ice box	+±	++	++	+	0	0	f. tr.	0	±	±	tr.	0

TABLE I, *b*

To 0.2 cc. of the diluted antigens were added 4 capillary drops of *l*-immune serum.

Readings taken after:	*l*-Antigen Dilution 1:				*d*-Antigen Dilution 1:				*m*-Antigen Dilution 1:			
	20	100	500	2500	20	100	500	2500	20	100	500	2500
1 hr. at room temperature	+	+±	+	tr.	0	0	0	0	f. tr.	tr.	f. tr.	0
3 hrs. at room temperature	+	++	+±	±	0	0	0	0	tr.	tr.	tr.	0
Night in ice box	++±	+++	++±	+	0	f. tr.	0	0	±	+	tr.	0

TABLE II, *a*

To 0.2 cc. of the diluted antigens were added 2 capillary drops of *d*-immune serum.

Readings taken after:	*l*-Antigen Dilution 1:				*d*-Antigen Dilution 1:				*m*-Antigen Dilution 1:			
	20	100	500	2500	20	100	500	2500	20	100	500	2500
1 hr. at room temperature	0	0	0	0	tr.	+	+	tr.	0	tr.	f. tr.	0
3 hrs. at room temperature	0	0	0	0	tr.	+	+	±	f. tr.	tr.	f. tr.	0
Night in ice box	0	0	f. tr.	0	±	++	++	+±	±	±	tr.	0

TABLE II, *b*

To 0.2 cc. of the diluted antigens were added 4 capillary drops of *d*-immune serum.

Readings taken after:	*l*-Antigen Dilution 1:				*d*-Antigen Dilution 1:				*m*-Antigen Dilution 1:			
	20	100	500	2500	20	100	500	2500	20	100	500	2500
1 hr. at room temperature	0	0	0	0	+	+±	+	tr.	tr.	tr.	f. tr.	0
3 hrs. at room temperature	0	0	0	0	+	+±	+	tr.	tr.	tr.	tr.	0
Night in ice box	0	0	0	0	++±	+++	++±	+	+	+	tr.	0

TABLE III, *a*

To 0.2 cc. of the diluted antigens were added 2 capillary drops of *m*-immune serum.

Readings taken after:	*l*-Antigen Dilution 1:				*d*-Antigen Dilution 1:				*m*-Antigen Dilution 1:			
	20	100	500	2500	20	100	500	2500	20	100	500	2500
1 hr. at room temperature	0	0	0	0	0	0	0	0	±	+	+	±
3 hrs. at room temperature	0	0	0	0	0	0	0	0	±	+	+±	±
Night in ice box	0	0	f. tr.	0	0	0	0	0	++	++±	++±	+±

TABLE III, *b*

To 0.2 cc. of the diluted antigens were added 4 capillary drops of *m*-immune serum.

Readings taken after:	*l*-Antigen Dilution 1:				*d*-Antigen Dilution 1:				*m*-Antigen Dilution 1:			
	20	100	500	2500	20	100	500	2500	20	100	500	2500
1 hr. at room temperature	0	0	0	0	0	0	0	0	±	+	+	tr.
3 hrs. at room temperature	0	0	0	0	0	0	0	0	±	++	+±	±
Night in ice box	0	f. tr.	f. tr.	0	0	0	0	0	++±	+++	+++	+

TABLE IV, *a*

l-Antigen and l-Immune Serum

Readings taken after:	1*	2	3	4	5	6	7	8	9	10	11	12	Control
15 mins. at room temperature	f. tr.	±	tr.	0	±	f. tr.	+	±	±	+	+	+	+
3 hrs. at room temperature	±	+	±	0	+	±	+±	+	+	+±	+±	+±	+±
Night in ice box	+	++	+	0	+	±	++	+±	+±	++	++	++	++

TABLE IV, *b*

d-Antigen and d-Immune Serum

Readings taken after:	1	2	3	4	5	6	7	8	9	10	11	12	Control
15 mins. at room temperature	±	0	tr.	tr.	0	f. tr.	+	±	tr.	±	+	+	+
3 hrs. at room temperature	+	±	+	±	0	tr.	+±	+	±	+±	+±	+±	+±
Night in ice box	+±	+	++	++	0	±	++	++	+±	++±	++±	++±	++±

TABLE IV, *c*

m-Antigen and m-Immune Serum

Readings taken after:	1	2	3	4	5	6	7	8	9	10	11	12	Control
15 mins. at room temperature	+	+	tr.	tr.	±	0	+	±	±	±	±	+	+
3 hrs. at room temperature	+±	+±	±	+	+	0	+±	+±	+±	+±	++	+±	++
Night in ice box	++±	++±	+±	++±	++	0	++±	++	++	+++	++±	+++	++±

*The numbers 1 to 12 refer to the substances tested for inhibition.

comparison was tested. The compounds are enumerated as follows: (1) levo-tartaric acid, (2) dextro-tartaric acid, (3) meso-tartaric acid, (4) levo-paraaminotartranilic acid, (5) dextro-paraaminotartranilic acid, (6) meso-paraaminotartranilic acid, (7) succinic acid, (8) l-malic acid, (9) d-l-malic acid, (10) lactic acid, (11) benzoic acid, (12) acetic acid.

0.2 cc. of the antigen (diluted 1:500) were mixed with 0.05 cc. of a neutral solution containing 1 millimol in 10 cc. of the substances indicated. To this 4 capillary drops of the homologous immune serum were added. The control tube contains only antigen and immune serum.[1]

TABLE V

The inhibiting substances used were: (4) *l*-paraaminotartranilic acid, (5) *d*-paraaminotartranilic acid, (6) *m*-paraaminotartranilic acid, (13) *l*-paranitrotartranilic acid, (14) *d*-paranitrotartranilic acid.

	Readings taken after	4	5	6	13	14	Control
l-antigen and *l*-immune serum	15 mins. at room temperature	0	±	tr.	0	±	+
	3 hrs. at room temperature	0	+	±	0	+	+±
	Night in ice box	0	+±	+±	0	+±	++
d-antigen and *d*-immune serum	15 mins. at room temperature	±	0	tr.	tr.	0	+
	3 hrs. at room temperature	+	0	±	±	0	+±
	Night in ice box	+±	0	+±	+±	0	+±
m-antigen and *m*-immune serum	15 mins. at room temperature	±	±	0	tr.	±	±
	3 hrs. at room temperature	+	+	0	+	+	+±
	Night in ice box	+±	+±	0	+±	+±	++

Table V represents inhibition tests with *l*-, *d*- and *m*-paraamino-tartranilic acid and *l*- and *d*-paranitrotartranilic acid where 0.05 cc. of a neutral solution containing in 10 cc. 0.25 millimol of these substances was added to the antigen. The *m*-paranitrotartranilic acid could not be used since a precipitation occurred on mixing with saline solution.

DISCUSSION AND SUMMARY

The experiments reported confirm the results of our previous studies and demonstrate again the striking influence of the steric constitution

[1] In the details given for the tests in a previous paper (1), Table IV *c*, it should read "0.5 millimol in 10 cc."

on serological properties. It would seem that in this respect the specificity of serum reactions is analogous to that of ferments. However, while in the investigations with ferments one is limited to those found in nature, by our method antibodies can be produced at will, which act on chosen substances.

In particular one sees that the change in the spatial configuration with regard to one asymmetric carbon atom is sufficient to cause a pronounced serological difference, as appears from a comparison of the reactions of either the *l*- or *d*-antigen with those of the *m*-antigen. Since the change of the levo- into the dextro-acid would involve a rearrangement of the groups around both asymmetric carbon atoms one may suppose that there is a greater serological difference between these two acids than between either and the *m*-tartaric acid. This reasoning is supported by the fact that the *l*-immune sera showed almost no reactions on the *d*-antigen and vice versa while there were weak group reactions of the *l*- and *d*-immune sera with the *m*-antigen. A question which requires further investigation is whether the marked serological distinction between steric isomers depends on the nature of the radicals connected with the asymmetric carbon atom, *i.e.*, whether any radical will produce an effect of the same order as polar groups like COOH or OH (*c.f.* Reiner (5)).

The fact that the specificity of the reactions is entirely determined by the substances linked to the protein and not by the protein molecule itself is in the first place due to the method of testing, namely, the use of a protein different from that employed for immunization. Besides one has to consider that, owing to the considerable number of tyrosine and histidine groups with which the azocompounds can combine, one may visualize the protein molecules, studded, as it were, with the groups artificially introduced.

The application of the findings presented, to the problem of specificity in natural antigens, especially those of carbohydrate nature, is suggested because the chemical constitution of tartaric acid is closely allied to that of the sugar acids which, from the work of Avery, Heidelberger and Goebel are known to form essential parts of the bacterial carbohydrate haptens. Since these haptens are high molecular compounds consisting of structurally different combinations of sugars and various carbohydrate acids it is easily conceivable

that there exists an almost unlimited number of such specific substances.

A special point is brought out by the inhibition tests. These tests distinguish definitely between the uncombined *l*- and *d*-tartaric acids themselves, while in contrast to the direct precipitin reactions there is no definite distinction between *l*- or *d*- and the *m*-tartaric acid, in the tests with *l*- or *d*-immune serum. The *m*-immune serum however differentiates clearly the *m*-tartaric acid from the two other acids. In comparison with the simple tartaric acids a considerably greater inhibiting effect and a more marked specificity similar to that of the precipitin tests is exhibited by the amino- or nitrotartranilic acids. This indicates that the antibodies involved do not act upon the tartaric acid part of the molecule only but attach themselves also to the aromatic nucleus and hence may be supposed to possess several distinct binding groups. This view suggests a further study of substances of complicated chemical structure.

In the present connection it is pertinent to state that in general the conclusions drawn from the inhibition tests are based upon reactions with substances whose chemical constitution is known in its entirety, and that of course proteins play no part whatsoever in the specificity of these phenomena.

SUMMARY

In continuation of studies on the stereochemical specificity of serum reactions, antigens were examined containing the acyl radicals of the levo-, dextro-, and meso-tartaric acids. It was found that in this case also, immune sera can readily be obtained which differentiate sharply the three antigens identical in every other respect but possessing stereoisomeric groups.

Since the tartaric acids by their chemical constitution belong to the same class of substances as sugar acids the results have a bearing upon the question of the specificity of natural antigens containing carbohydrates such as have been described by Avery and Heidelberger.

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THE ANTIBODY-FORMATION BY POLYSACCHARIDS

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That antibodies are formed for lipoids had long been doubted. Recently, however, this doubt has been cleared away by the production of the antibodies (1) (2) in animals injected with lipoids and serum proteins.

It has occurred to me that polysaccharids, which belong to the same sort of emulsoids, might be endowed with antigenic properties and that their action as antigens might be facilitated by using serum proteins or other substances as vehicle.

The fact that glycolytic ferment develops in the animal body after the injection of sugar has been demonstrated by Weinland, Abderhalden, and others. There is, however, little literature dealing with the antigenic nature of sugar. Heidelberger and Avery (3) (4) have isolated a substance which bore close resemblance to polysaccharids from the pneumococci. This substance precipitated antipneumococcic serum, but immunization experiments with it have given a negative result. Rokuro Kondo (5) obtained an antilipoidal serum by the use of a mixture of lipoids with inulin.

I have experimented with inulin, soluble starch and dextrine, and succeeded in obtaining antisera against all of them. The results were reported in the December issue of the *Japan Medical World*, Home Edition in 1927 (6), and in the *Saikingaku Zasshi* (*Journal of Bacteriology*) April, 1928 (7). I shall deal with the same matter in this report, reviewing all I have reported previously in addition to what I have done since.

Methods

The three kinds of polysaccharids used in my experiments were of Merck make. I prepared solutions of them and injected them into the postauricular veins of rabbits daily for some time. On the 5th to 6th day from the last injection the blood of the animals was collected, and the serum was separated and inactivated

at 56°C. for ½ hour. The test was made by complement fixation and precipitation, using the corresponding polysaccharids as antigens.

Complement Fixation Test.—The serum was put in a series of 6 test tubes containing 0.2, 0.1, 0.05, 0.025, 0.0125 and 0.006 cc. of it respectively, made to 0.5 cc. with saline. Tube 1 was a control, containing serum only. To the remaining 5 tubes 0.5 cc. of the antigen was added. The complement consisted of 0.5 cc. of diluted guinea-pig serum, containing 2.5 lytic units. The mixtures were kept for 1 hour in the incubator, and then 1 cc. of the hemolytic system was added (0.5 cc. of a 5% goat erythrocytal suspension and 0.5 cc. of the anti-sheep serum having 3 times as strong a hemolytic power) incubation done for 2 hours more and the results were read.

The inulin, used as antigen in the complement fixation test was a 2% solution, the soluble starch a 1.5% solution, and the dextrine a 3% solution in saline. All were dissolved by heating. Each of these antigen-solutions (1.5 cc.) was tested and found to give no auto-inhibition.

EXPERIMENTAL

A. Inulin

Material for Injection.—Inulin was dissolved in saline to the proportion of 4% and heated at 70°C. Prior to injection, 0.8 cc. of the solution was mixed either with 0.2 cc. of the pig serum or with the same amount of the saline. The mixture was then put in the incubator for 1 hour. The controls received pig serum only, in the same dilution.

Doses and Number of Injections.—Each of the three mixtures described above was injected in a dose of 1 cc., 1.5 cc., 2 cc., 3 cc., 4 cc., 5 cc., and 6 cc., each dose being repeated.

Complement Fixation Test.—The normal serum often gave a very weak positive reaction. These positive sera, however, gave a negative reaction when they were inactivated by heating at 60°C. for 1 hour. They also gave a negative reaction when the mixture was allowed to stand at 0°C. for 1½ hours instead of at 37°C. before the hemolytic system was added. From these facts it will be seen that the positive reaction, which the normal sera gave, was non-specific in nature.

Results of complement fixation tests with the sera of injected rabbits are given in Table I.

From Table I, it will be seen that the sera of the rabbits, which had been treated either with inulin solution alone or with inulin-pig-serum mixture, all gave positive results. The control serum from the rabbit injected with the pig serum alone, gave a negative reaction.

Precipitation Tests.—These were carried out by both the mixing and the ring precipitation methods, but both yielded negative results.

Bunji Imai (8) has reported that by the complement fixation test he has demonstrated the antigenic power of inulin. He also obtained immune sera, which gave precipitation with a certain kind of inulin.

B. Soluble Starch

All the immunization experiments with a weak starch solution proved unsuccessful, but by the use of a strong one, the antibody formation was at last induced.

TABLE I

Results of the Complement Fixation Test with the Sera of the Rabbits Receiving Inulin Only

Rabbit No.	Serum (cc.)					
	0.2	0.1	0.05	0.025	0.0125	0.006
1	L	H	H	H	H	K
2	L	H	H	H	H	k
3	L	H	H	K	L	L
4	L	H	K	L	L	L
5	L	H	K	L	L	L
6	L	H	K	L	L	L

Results of the Complement Fixation Test with the Sera of the Rabbits Receiving Inulin Plus Pig Serum

7	K	H	H	K	k	L
8	k	H	H	H	K	k
9	L	H	H	H	K	L
10	H	H	H	H	K	L
11	H	H	H	H	H	K

H stands for inhibition, K or k incomplete hemolysis and L complete hemolysis in the complement fixation.

Material for Injection.—A 3% soluble starch saline mixture was heated and made into a pasty substance. It was used without any vehicle.

Doses and Number of Injections.—A daily injection with a dose of 0.5 cc. was given for 18 days.

Complement Fixation Test.—There was no case in which normal serum gave a positive result. After the completion of one course of injections, the sera of all the treated animals gave positive results, as shown in Table II.

Precipitation Test.—The tests all gave negative results.

Ken Nodzu (9) as well as B. Imai (8) have demonstrated the antigenic power of soluble starch by the complement fixation test. Nodzu

TABLE II

Results of the Complement Fixation Test with the Serum of the Rabbits Receiving Soluble Starch

Rabbit No.	Serum (cc.)					
	0.2	0.1	0.05	0.025	0.0125	0.006
12	L	H	H	K	L	L
13	L	K	L	L	L	L
14	L	H	K	L	L	L
15	L	H	H	H	K	L
16	L	H	K	L	L	L

TABLE III

Results of the Complement Fixation Test with the Serum of the Rabbits Receiving Dextrine Only

Rabbit No.	Serum (cc.)					
	0.2	0.1	0.05	0.025	0.012	0.006
17	L	K	L	L	L	L
18	L	K	L	L	L	L
19	L	K	k	L	L	L
20	L	K	k	L	L	L
21	L	L	L	L	L	L
22	L	L	L	L	L	L

Results of the Complement Fixation Test with the Serum of the Rabbits Receiving Dextrine Plus Pig Serum

23	K	H	H	H	H	K
24	K	H	K	L	L	L
25	K	H	K	L	L	L
26	L	H	H	H	K	L
27	K	H	H	H	H	K
28	L	H	H	H	K	L
29	K	H	H	H	H	K
30	L	H	H	K	L	L

stated that he, like myself, obtained only negative precipitation tests, but Imai states that he obtained a weakly positive reaction.

C. Dextrine

Experiment 1

Material.—The dextrine used in my experiments was a white powder, which gave a purplish indigo color with iodine. It was insoluble except when heated; and therefore must have been amylodextrine. (For convenience sake it is stated as "D.I.") A 6% mixture of D.I. with the saline was made by heating at 70°C. The pig serum was added in the same proportion as in the case of inulin.

Dose and Number of Injections.—These were the same as in the case of inulin.

Complement Fixation Tests.—The normal sera gave positive reactions less frequently than in the case of inulin, and gave less marked reactions. By differential testings, the reaction was proved to be nonspecific.

The results of the complement fixation tests with the sera of rabbits treated with dextrine alone or with the mixture of dextrine and pig serum are shown in Table III. It will be seen that the serum of the rabbits immunized with the mixture of dextrine and pig serum gave a strong reaction, whereas those from animals receiving dextrine alone yielded doubtful reactions. The control serum from the rabbit injected with pig serum alone, gave a negative reaction.

Precipitation Reaction.—The results were entirely negative. Thus far, dextrine had induced antibodies only when injected in association with pig-serum. Because of the fact, however, that inulin and soluble starch gave antibodies even though injected without the addition of pig-serum, I next submitted animals to an increased amount and number of injections, in order to determine whether or not the addition of pig-serum is absolutely necessary to effect the antibody formation.

Experiment 2

Material.—The kind of dextrine and the concentration of its solution were the same as employed in Experiment 1.

Dose and Number of Injections.—18 injections with a dose of 5 cc. were given.

Complement Fixation Tests.—The results of the complement fixation tests made with the sera of the treated animals are shown in Table IV. It will be seen that dextrine when given by itself elicited antibodies.

Experiment 3

Material.—I used two different kinds of Merck dextrine, both being further decomposed than that of the previous experiments. One was a white powder given a purple color with iodine, and easily soluble. (For convenience sake this

lot is called "D.II.") The other was a yellow granular substance giving a red color with iodine and also easily soluble. (This is called "D.III.") D.III. was found to be erythrodextrine. In degree of decomposition, D.II. stood just between D.I. and D.III. The D.II. and D.III. specimens were dissolved in saline to a proportion of 6%, and injected into animals without the addition of the pig serum.

Amount and Number of Injections.—18 injections were given with a dose of 5 cc.

Complement Fixation Test.—All the sera gave negative results.

Experiment 4

Material.—A 6%, 10% and 15% saline solution of the D.II. and D.III. were prepared.

Amount and Number of Injections.—One course of treatment consisted of 5 injections each of 6%, 10% and 15% solutions in saline, the dose being 10 cc. The total amount of the injected material was, therefore, about three times as much as it was in the previous experiments.

TABLE IV

Results of the Complement Fixation Test with the Serum of the Rabbits Receiving Dextrine Only

Rabbit No.	Serum (cc.)					
	0.2	0.1	0.05	0.025	0.012	0.006
31	L	H	H	K	L	L
32	L	H	K	L	L	L
33	L	H	H	L	L	L
34	L	H	H	K	L	L

Complement Fixation Test.—The results of the complement fixation test with the sera of the animals treated with D.II. and D.III. were all negative. It may be inferred that these two kinds of dextrine were lacking in antigenic action.

From the results of my experimental immunization with dextrine, it might be concluded that the results of Imai, as stated above, who could not successfully immunize the animal with seven repeated injections of 5 cc. of a 6% dextrine solution as such or mixed with 0.5 cc. of the inactivated pig serum, were due either to an insufficient number of injections or the use of an unsuitable kind of dextrines.

Specificity of the Antibody

After heating the immunized sera at 60°C. for 1 hour, no changes in the complement fixation reaction occurred. Nor was the reaction

influenced by carrying out the first half of the procedure of the test at 0°C. for ½ hour. From these facts, it may be concluded that the reaction is of a specific nature.

In order to determine the specificity of the immune serum reaction against the above mentioned three polysaccharids, I carried out cross tests with them. In this experiment, I used a 1.5% solution of the three polysaccharids as antigen. The results are shown in Table V. Each gave a specific reaction.

TABLE V

Results of the Complement Fixation Tests for the Specificity of the Immune Bodies Derived from Polysaccharids

Antigen	Soluble starch				Inulin				Dextrine (D.I.)			
Amount of sera cc.	0.1	0.05	0.025	0.012	0.1	0.05	0.025	0.012	0.1	0.05	0.025	0.012
Soluble starch immune rabbit serum:												
No. 15	H	H	H	K	L	L	L	L	L	L	L	L
No. 12	H	H	K	L	L	L	L	L	L	L	L	L
No. 16	H	K	L	L	L	L	L	L	L	L	L	L
Inulin immune rabbit serum:												
No. 11	L	L	L	L	H	H	H	K	L	L	L	L
No. 10	L	L	L	L	H	H	K	L	L	L	L	L
No. 5	L	L	L	L	H	K	L	L	L	L	L	L
Dextrine (D.I.) immune rabbit serum:												
No. 29	L	L	L	L	L	L	L	L	H	H	H	K
No. 30	L	L	L	L	L	L	L	L	H	H	K	L
No. 31	L	L	L	L	L	L	L	L	H	K	L	L

It may be noted here that the D.I. immune serum did not give any positive reaction to D.II. or D.III.

Very recently, Ken Nodzu (10) immunized an animal with starches from Indian corn, barley, wheat, potato, etc., and Yoshio Masuda (11) with those from two varieties of rice. Each of the immune sera reacted most strongly against that kind of starch which had been used as the antigen, while against others the reaction was very weak. The authors, therefore, insisted that they could immunologically differentiate starches, which it is very difficult to do by morphological features.

Fate of the Antibodies in the Animal Body

Blood was collected from the immunized rabbits at the end of every week and the complement fixation test was carried out with sera heated at 60°C. for 1 hour. The results are shown in Tables VI, VII and VIII.

TABLE VI

Results of the Complement Fixation Test with the Sera of Rabbits into Which Inulin Was Injected

Day of collection of blood	Results of the complement fixation with		
	Serum of Rabbit 10	Serum of Rabbit 11	Serum of Rabbit 5
1st week	H H H K L L	H H H H H K	L H K L L L
2nd week	L K k L L L	L H H H K L	L K L L L L
3rd week	L K L L L L	L H H K L L	L L L L L L
4th week	L L L L L L	L H H L L L	
5th week		L H L L L L	
6th week		L L L L L L	

TABLE VII

Results of the Complement Fixation Test with the Sera of Rabbits into Which Soluble Starch Was Injected

Day of collection of blood	Results of the complement fixation with		
	Serum of Rabbit 12	Serum of Rabbit 13	Serum of Rabbit 14
1st week	L H H K L L	L K L L L L	L H K L L L
2nd week	L H K k L L	L L L L L L	L K L L L L
3rd week	L K L L L L		
4th week	L L L L L L		

TABLE VIII

Results of the Complement Fixation Test with the Sera of Rabbits into Which Dextrine Was Injected

Day of collection of blood	Results of the complement fixation with		
	Serum of Rabbit 29	Serum of Rabbit 30	Serum of Rabbit 28
1st week	K H H H H K	L H H K L L	L H H H K L
2nd week	L H K L L L	L K K L L L	L H K L L L
3rd week	L K L L L L	L L L L L L	L H K L L L
4th week		L L L L L L	L K L L L L
5th week			L L L L L L

It will be seen that the sera in the period following the immunization lost their strength rapidly, those of animals weakly immunized giving a negative result in a fortnight, while the strongly immunized gave one in 6 weeks.

Do the Polysaccharids Themselves Elicit the Formation of Antibodies?

The above mentioned polysaccharids gave rise to antibodies, even when unassociated with vehicle. But is it the polysaccharids as such that cause the antibodies to develop, or may a trace of proteins co-mingled in the materials play the part of vehicle? To settle the matter I have carried out an experimental investigation.

In the first place, I made Millon's and biuret tests upon the three kinds of polysaccharid solutions. They all gave a negative result. I then tried quantitative estimation of the nitrogen in the specimens after Kjeldahl's microestimation method. The results were that inulin contained 0.036%, soluble starch 0.017% and dextrine (D.I.) 0.019% of nitrogen.

Are the Nitrogen Contents Proteins?

In order to find whether the nitrogenous substances are proteins the following experiments were carried out:

To 200 cc. of a 2% hydrochloric acid solution 20 gm. of soluble starch was added, and the mixture was hydrolyzed by being boiled for 3 hours. The liquid was then neutralyzed and subjected to the iodine reaction test. It proved negative. It was, therefore, certain that all the starch had been converted into glucose. For the purpose of removing the salts and glucose, some of the liquid was then put into a bladder and left in running water for 48 hours. Then, the remaining liquid in the bladder was evaporated to 1/10 volume. The concentrate had a blackish brown color, one so dark that it was impossible to make color reaction tests with it. It was found, however, to contain 0.01% of nitrogen on quantitative estimation. The liquid gave also a positive reaction of Heller's ring test.

Another portion of the hydrolyzed solution, which had been neutralized, was allowed to evaporate to 1/10 without being dialyzed and a quantitative estimation was made of its nitrogen contents. It was found to contain 0.011% of nitrogen. This liquid also gave a positive result with Heller's ring test.

From the results of the above described experiments, it seems certain that the nitrogen found in the soluble starch solutions was protein.

Is the nitrogen, which is found in inulin or dextrine, of protein origin?

This theme remains to be studied. It will probably be hard to free these polysaccharids perfectly from nitrogenous substances.

From all the accessible data it might be inferred that it would be difficult to determine whether or not proteins play an important part as the vehicle in the immunization processes, but from the following two facts, proteins are suspected to be intimately related with the antibody production by polysaccharids.

1. Considering the connection between concentration, dose and number of injections of the used polysaccharid solutions and also the amount of the produced antibodies, it might be judged that inulin gives rise to antibodies comparatively easily. However, it is hard to obtain immunization, with soluble starch and dextrine. The nitrogen contents of the latter two is only about one half that of the first.

2. Comparing the results of the complement fixation tests with the immune sera of rabbits injected with inulin and dextrine with and without pig-serum, it will be seen that while there did not occur any differences with inulin, which is relatively rich in nitrogen, with dextrine, which is poor in nitrogen, the serum of the animal treated with the mixture of dextrine and pig-serum gave by far the stronger result. (See Tables III and IV.)

The facilities for successful immunization depend largely upon the natural characteristics of the materials injected and the nature of the animals, yet from the above mentioned two facts it may well be thought that there is an intimate connection between the production of immune bodies for polysaccharids and the association with them of proteins.

The possibility arises that the antibodies demonstrated by the complement fixation test, might have been directed against incidental protein. This is rendered rather unlikely by the following considerations.

1. As described above, the dialyzate obtained from the hydrolyzation products of the starch solution yielded an absolutely negative iodine reaction, but was positive to Heller's test. However, the complement fixation test made with this dialyzate and the anti-starch serum proved negative.

2. The polysaccharid solutions, which were used as antigens, gave no color reaction of proteins. Their nitrogen contents was at a

minimum and probably was not protein. Granting all of it to have been such, the total amount of nitrogen found in the material employed as antigen in the complement fixation test was extraordinarily small. On calculation the proteins in 0.5 cc. of the polysaccharid solutions, which I employed in my test, would be about 8/1,000,000 gm. in the starch specimens and 20/1,000,000 gm. in those of inulin. The real protein contents was doubtless but a fraction of these calculated protein contents. It is unlikely that these traces should produce such a remarkable complement fixation reaction as I have obtained.

CONCLUSIONS

1. By complement fixation tests, it has been clearly demonstrated that the sera of rabbits immunized with inulin, soluble starch and dextrine contain specific antibodies.
2. All these immune sera gave a negative precipitation reaction.
3. The kind of dextrine which has a construction very near to starch has an antigenic property, but those in a state of further decomposition do not give rise to antibodies.
4. All the three kinds of polysaccharids have power to produce antibodies without any vehicle. Dextrine is the only one of the three that gives rise to immune bodies more readily when pig serum is added to it.
5. Regarded as antigens, inulin stood first and soluble starch and dextrine next in order.
6. All three kinds of polysaccharids that were employed gave a negative protein color reaction. All of them, however, contained nitrogen. It has been proved that the large portion of the nitrogen contained in the soluble starch is derived from its protein contents.
7. It is suggested that in the production of immune bodies by these three kinds of polysaccharids, proteins might play the part of the vehicle. This is, however, still to be determined.

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THE ANTIBODY RESPONSE IN THE HUMAN BEING AFTER INJECTION WITH NORMAL HORSE SERUM

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During the course of an investigation of the antibody response in persons treated with immune sera (1–2), studies were carried out upon the sera of three patients who had received 50 cc. of normal horse serum by intramuscular or subcutaneous injection and where bleedings had been secured at close intervals. In all these cases, in contrast to those which had received immune sera, there was found a failure of antibody response and even a minor skin sensitiveness—in spite of the large dosage. Serum sickness had also failed to appear in these cases, and it was to this that the absence of circulating antibodies was attributed, since their production has been found, usually, to be correlated with the appearance of this phenomenon (1–4).

To test this assumption, sera were studied in cases of mild skin lesions receiving varying amounts of normal horse serum as a form of non-specific protein therapy. The study was carried out in the same manner as in that previously reported (1). The patients were bled prior to the injection and at various intervals, approximately the same as in the immune serum cases, thereafter; and exactly the same procedures were carried out in the determination of the various types of antibodies: precipitins, anaphylactic antibodies, reagins and the transferred guinea pig skin reaction.

A total of 91 sera from eighteen patients were thus studied. Normal horse serum, without preservative, obtained in four different lots from the pooled blood of six to eight normal horses was used. Skin tests to horse serum, in a dilution of 1:10, made before treatment, were uniformly negative. Two of the patients received 100 cc. by intravenous injection; two, 50 cc. by intramuscular; one, 50 cc. by subcutaneous; seven, 50 cc. by intravenous; five, 20 cc. by intravenous; and one, 10 cc. by intravenous. Serum sickness occurred in fifteen of the eighteen cases, (83%), in this group—an incidence about the same as that found in our series of immune serum treated cases.

TABLE I

Anti-Body Response in Cases Treated with Normal Horse Serum

Case No.		Treatment	Interval after treatment	Serum sickness	Skin reaction	Prausnitz-küstner	Guinea pig ear reaction	Anaphylactic antibodies	Precipitins
1.	I	10 cc., intravenous	Before	—	Neg.	Neg.	±	—	—
	II		14 days	—	Neg.	—	Neg.	Neg.	Neg.
	III		21 days	—	Neg.	—	—	—	—
2.	I	20 cc., intravenous	Before	—	Neg.	Neg.	Neg.	Neg.	Neg.
			10 days	Very mild					
	II		14 days		++	—	Neg.	—	1:10, +
3.	I	20 cc., intravenous	Before	—	Neg.	Neg.	—	—	—
			5 days	Moderately severe, 3 days					
	II		9 days		±	Neg.	—	—	Neg.
	III		16 days		++	Neg.	++	Neg.	Neg.
	IV		23 days		++	—	Neg.	Neg.	—
4.	I	20 cc., intravenous	Before	—	+	—	Neg.	Neg.	—
			10 days	Moderately severe, 3 days	+	Neg.	Neg.	Neg.	Neg.
	II		19 days		±	Neg.	Neg.	Neg.	Neg.
	III		28 days		+	Neg.	+++	Neg.	Neg.
5.	I	20 cc., intravenous	Before	—	Neg.	—	Neg.	Neg.	—
			8 days	Marked, 4 days					
	II		22 days		++	Neg.	Neg.	Neg.	—
6.	I	20 cc., intravenous	Before	—	Neg.	Neg.	Neg.	Neg.	Neg.
			10 days	Moderately severe, 3 days					
	II		14 days		+	Neg.	Neg.	Neg.	1:10, ±
	III		17 days		+	Neg.	—	Neg.	Neg.
	IV		21 days		++	Neg.	+	Neg.	Neg.
	V		28 days		++	Neg.	+	Neg.	Neg.
7.	I	50 cc., intravenous	Before	—	Neg.	Neg.	Neg.	Neg.	—
	II		7 days	—	Neg.	Neg.	Neg.	Neg.	—
			11 days	Severe, 5 days					
	III		12 days		++	Neg.	Neg.	Neg.	—
	IV		16 days		+++	Neg.	Neg.	Neg.	—
	V		21 days		+++	Neg.	Neg.	Neg.	—
	VI		24 days		+++	Neg.	Neg.	Neg.	Neg.
	VII		31 days		++	Neg.	Neg.	Neg.	Neg.

TABLE I—*Continued*

Case No.	Treatment	Interval after treatment	Serum sickness	Skin reaction	Prausnitz-küstner	Guinea pig ear reaction	Anaphylactic antibodies	Precipitins
8. I	50 cc., intravenous	Before	—	Neg.	Neg.	Neg.	Neg.	—
		6 days	Marked, about 5 days					
II		7 days		±	Neg.	+	Neg.	—
III		20 days		++	Neg.	+++	Neg.	—
IV		34 days		++	Neg.	—	—	Neg.
9. I	50 cc., intravenous	Before		Neg.	Neg.	±	Neg.	—
		Same day	Marked local reaction					
II		7 days		Neg.	Neg.	Neg.	Neg.	Neg.
III		14 days		±	Neg.	++	Neg.	—
IV		18 days		±	Neg.	Neg.	Neg.	—
10. I	50 cc., intravenous	Before	—	Neg.	Neg.	Neg.	Neg.	—
II		7 days	—	Neg.	Neg.	Neg.	±	—
		8 days	Severe, 4 days					
III		14 days		+	Neg.	++	—	—
IV		21 days		+	—	—	Neg.	—
V		35 days		+	Neg.	Neg.	Neg.	Neg.
11. I	50 cc., intravenous	Before	—	Neg.	Neg.	Neg.	Neg.	—
II		7 days	—	Neg.	Neg.	Neg.	Neg.	—
		10 days	Moderately severe, 8 days					
III		14 days		±	Neg.	+	Neg.	Neg.
IV		21 days		+++	Neg.	Neg.	Neg.	—
V		29 days		++++	Neg.	Neg.	Neg.	—
VI		39 days		++++	Neg.	Neg.	Neg.	—
12. I	50 cc., intravenous	Before	—	Neg.	±	±	Neg.	—
		6 days	Moderately severe, 2 days					
II		9 days		+	Neg.	—	Neg.	Neg.
III		16 days		+	Neg.	Neg.	Neg.	—
IV		30 days		±	Neg.	±	Neg.	Neg.
13. I	50 cc., intravenous	Before	—	Neg.	Neg.	Neg.	Neg.	—
		6 days	Marked, 4 days					
II		7 days		Neg.	Neg.	±	Neg.	Neg.

TABLE I—*Continued*

Case No.	Treatment	Interval after treatment	Serum sickness	Skin reaction	Prausnitz-küstner	Guinea pig ear reaction	Anaphylactic antibodies	Precipitins
13. III		9 days		Neg.	Neg.	+	Neg.	—
IV		14 days		++	Neg.	Neg.	Neg.	Neg.
V		17 days		++	Neg.	Neg.	Neg.	—
VI		27 days		++	Neg.	Neg.	Neg.	—
14. I	50 cc., intramuscular	Before	—	±	Neg.	Neg.	Neg.	—
		Same day	Urticaria and marked local					
II		7 days		Neg.	Neg.	+	Neg.	—
		10 days	Severe, 4 days					
III		15 days		++	Neg.	Neg.	Neg.	Neg.
IV		23 days		+	Neg.	++	Neg.	Neg.
V		30 days		++	Neg.	±	Neg.	—
VI		41 days		++	Neg.	Neg.	Neg.	Neg.
VII		53 days		++	Neg.	—	—	—
VIII		74 days		++	Neg.	—	—	—
15. I	50 cc., intramuscular	Before	—	Neg.	Neg.	±	Neg.	—
		Same day	Marked local					
II		9 days		Neg.	Neg.	+	Neg.	—
		10 days	Severe, 4 days					
III		17 days		++	+	++	—	1:10, ±
IV		23 days		+++	+	Neg.	Neg.	1:10, ±
V		30 days		+++	±	+	Neg.	—
VI		38 days		++	Neg.	+	Neg.	—
VII		56 days		++	Neg.	+	Neg.	—
16. I	50 cc., subcutaneous	Before	—	Neg.	—	—	—	—
II		1 day	—	Neg.	—	Neg.	Neg.	—
III		5 days	—	Neg.	—	Neg.	Neg.	—
IV		10 days	—	++	Neg.	±	—	—
V		21 days	—	—	—	±	+	—
VI		29 days	—	++	Neg.	±	Neg.	—
17. I	100 cc., intravenous	Before	—	Neg.	Neg.	Neg.	Neg.	Neg.
II		7 days	—	Neg.	Neg.	++	Neg.	—
III		14 days	—	+	+	Neg.	±	1:10, ±
IV		20 days	—	+++	++	Neg.	Neg.	Neg.
V		24 days	—	++	++	Neg.	Neg.	Neg.
VI		38 days	—	++	+	Neg.	Neg.	—
VII		52 days	—	+++	+	Neg.	Neg.	—

TABLE I—*Concluded*

Case No.	Treatment	Interval after treatment	Serum sickness	Skin reaction	Prausnitz-küstner	Guinea pig ear reaction	Anaphylactic antibodies	Precipitins
18. I	100 cc., intravenous	Before	Immediate urticaria	±	Neg.	++	Neg.	Neg.
II		7 days		Neg.	Neg.	+	Neg.	Neg.
		11 days	Doubtful					
III		12 days		Neg.	Neg.	+	Neg.	—
IV		19 days		+	Neg.	+	Neg.	Neg.
V		26 days		++	Neg.	Neg.	Neg.	Neg.
VI		32 days		++	Neg.	—	—	—
VII		35 days		+	Neg.	Neg.	Neg.	Neg.
VIII		54 days		+	Neg.	Neg.	Neg.	Neg.

The results of the antibody studies of these sera are shown in Table I. When the antibody response in this group is compared with that in which immune sera were used, the almost complete failure of antibody response to the injection of normal horse serum is striking, even though in several instances the amount injected was about the same as that usually used for treatment with immune sera. When we consider the antibodies in particular we find that the precipitins were always lacking or doubtful; anaphylactic antibodies were not found in any case; reagins were demonstrated with very weak reactions in two cases, one of which was a skin case with eczema (possibly allergic); skin sensitivity, though present, was certainly less marked than in the former group; and the guinea pig ear-skin reaction, which in the immune serum group was usually the most consistently demonstrable of all the reactions used,—the first to appear and the last to disappear,—was entirely negative, or weak, at best. In general, the findings for this group, when summarized and compared with those for sera in immune serum treated cases, indicate an almost complete failure in (circulating) antibody response, when normal horse serum is used for treatment.

That this failure may not be charged to variations in the conditions of the two experiments, the following observations are offered: Several lots of horse serum were used during the experiment, obviating any special antigenic value that might be ascribed to the serum of an

individual animal; serum sickness similar in every respect to that occurring after immune serum injection occurred, usually in a more or less severe form, in as great a percentage of cases as in the first experiment; finally the cases for both groups were equally unselected.

This failure to find circulating antibodies to horse serum, as such, after treatment with normal horse serum may be seen as presenting certain obstacles to that theory of the mechanism of serum sickness which would correlate the clinical manifestations with the presence of circulating antibodies. It is to be noted however, that the studies, upon which these theories have been based have been carried out with various types of immune sera; no reference has been found in the literature as to the use of normal horse serum for such a study as is here given.*

The relative merits of the various theories as to the basic mechanism of serum sickness have been adequately reviewed by von Pirquet and Schick (4) and by Coca (6); it is necessary only to point out, in view of a more or less complete failure of normal horse serum to call forth the production of the antibodies responsible for the Prausnitz-Küstner reaction, precipitins, the passive transfer of the anaphylactic reaction, and the transferred guinea pig skin reaction—as shown in the protocol—that the theory of a basic antigen-antibody reaction in serum sickness is further questioned. Subsequent investigation, however, may afford an explanation of the parodoxical findings here presented.

SUMMARY AND CONCLUSIONS

After the injection of normal horse serum in the human being, serum sickness occurs even more regularly than in cases treated with the various immune sera, but this is not accompanied by the production, to any notable degree, of circulating antibodies of the various types that are regularly to be demonstrated after the administration of immune serum and its resulting serum sickness.

Since normal horse serum therefore appears to be weakly antigenic, and immune serum highly antigenic for the human being, one must

* Dr. Coca, in a private communication as to unpublished data of his study of serum sickness among Indians (5) following the use of normal horse serum, reports that he was unable to demonstrate precipitins at any time.

assume that this difference is the result of some alteration in its antigenic characteristics produced during the course of the immunization or of its preparation for use; or that the specific antibody which is responsible for the phenomenon of serum sickness has not yet been identified; or that this phenomenon is not in any way dependent on the presence of the various known antibodies to normal horse serum.

The authors wish to express their appreciation of Dr. Jay Frank Schamberg's courtesy in furnishing access to clinical material, and to the Mulford Company for their generous donation of the serum used in the experiments.

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THE EFFECTS OF CATHODE RAYS ON THE PROTEINS OF SERUM

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Experiments conducted by Dr. Carrel in this laboratory revealed that chicken fibroblasts underwent a very characteristic change when cultivated in serum which had been submitted to irradiation by cathode rays. The cells resembled in appearance those of colonies cultivated in proteoses or peptones. Their cytoplasm was completely filled with granulations but, unlike degenerating cells, they maintained long, sharp, active pseudopods. They did not, however, undergo the rapid proliferation which is caused by partially hydrolyzed protein. On the other hand, they multiplied slightly more rapidly than control tissues cultivated in portions of the serum that had not been irradiated. These phenomena indicated that the cathode rays had produced certain chemical changes in the serum, probably in its protein constituents. The following study was made in an attempt to ascertain the nature of these changes.

The cathode rays were supplied by a Coolidge cathode ray tube,[1] operated at about 200 kilovolts (Tables I and II), and 0.2 milliamperes. The electrons did not penetrate more than a millimeter beyond the surface of the serum, so that the material was exposed either directly in the form of a thin layer, or in a cell behind a very thin mica window. In the latter case, much longer exposures were necessary, and the liquid was kept constantly stirred.

Fifteen cubic centimeter samples of chicken plasma were coagulated on Petri dishes of 180 sq. cm. area and exposed in a nearly vertical position 8 cm. from the window of the cathode ray tube. Exposures were made for 30 and 45 minutes. The serum was extracted from the irradiated plasma and also from control samples of coagulated plasma by grinding with sand. The cryoscopic points were determined and any necessary correction for evaporation was made. All samples were analyzed (1) for total nitrogen by the Pregl[2] micro method, (2) for amino nitrogen

[1] Coolidge, W. D., *J. Franklin Inst.*, 1926, **202**, 693.

[2] Pregl, F., Die Quantitative Organische Mikroanalyse. Berlin, 1923, 113.

by the Van Slyke[3] method, and (3) for non-protein nitrogen by precipitation with trichloracetic acid and determination of the nitrogen in the filtrate. There was a decided decrease in the concentration of protein nitrogen, the loss being from 32 to 42 per cent of the quantity originally present (Table I). There was a decrease in amino nitrogen corresponding to the loss of protein, and a small but significant increase in non-protein nitrogen of 15 to 42 mg. per 100 cc. (Table II). Since the protein which had disappeared did not appear in the non-protein fraction, it had evidently become insoluble and had been removed with the fibrin in transforming

TABLE I

Effect of Cathode Rays on the Concentration of Protein Nitrogen in Serum

Kind of serum used	Time of exposure	Voltage in kilovolts	Concentration of protein nitrogen			
			In control sera, mg. per 100 cc.	In irradiated sera, mg. per 100 cc.	Decrease	
					mg. per 100 cc.	per cent of original concentration
	min.					
Chicken serum from irradiated plasma						
1	30	210	474	273	201	42
2	45	210	399	243	156	39
3	45	195	664	454	210	32
Chicken serum irradiated as such						
	hrs.					
4	2½	160	555	510	45	8.1
Ox serum						
5	5	180	1,135	1,015	120	10.6
6	3	180	1,130	1,110	20	1.8
7	3	195	1,146	1,066	80	7.0
8	3	195	1,204	1,144	60	5.0

the plasma to serum. This supposition was substantiated in later experiments in which serum itself was irradiated.

A flat, vertical cell of about 20 cc. capacity was used for the irradiation of serum. The rays were admitted through a mica window 0.01 mm. thick and about 7 sq. cm. in area. Vigorous stirring was effected by means of a glass paddle. The time of irradiation varied from 2½ to 5 hours (Tables I and II).

Both ox serum and chicken serum were irradiated in the cell. The chemical changes observed were strictly analogous to those noted in the serum from the

[3] Van Slyke, D. D., *J. Biol. Chem.*, 1912, **12**, 275.

irradiated plasma (Tables I and II). During the course of the irradiation, a tough coating of yellowish-brown solid formed on the window of the cell. It was insoluble in dilute acids and alkalies, unchanged by alcohol and ether, but dissolved in concentrated nitric acid. The serum removed from the cell was distinctly opalescent, but no separation of solid material was obtained when it was centrifuged for a half hour, at 36,000 r. p. m. at 12 cm. radius.

The solid which collected on the window of the cell was dried and weighed. Analysis showed it to contain 11.8 per cent nitrogen. The decrease of nitrogen in

TABLE II

Effect of Cathode Rays on the Concentration of Non-Protein Nitrogen in Serum

Kind of serum used	Time of exposure	Voltage in kilovolts	Concentration of non-protein nitrogen		
			In control sera, mg. per 100 cc.	In irradiated sera, mg. per 100 cc.	Increase, mg. per 100 cc.
	min.				
Chicken serum from irradiated plasma					
1	30	210	66.0*	108.0*	42.0
2	45	210	26.9*	42.1*	15.2
3	45	195	92.0	107.0	15.0
Chicken serum irradiated as such					
	hrs.				
4	2½	160	10.5*	14.7*	4.2
Ox serum					
5	5	180	43	72	29
6	3	180	59	79	20
7	3	195	45	96	51
8	3	195	46	77	31

* Precipitation by trichloracetic acid.

the total amount of serum irradiated was equal, within the limits of experimental error, to that which separated in the solid material.

In order to ascertain whether the cathode rays were affecting the albumin or the globulin of the sera, samples were analyzed for their albumin and globulin contents by precipitation with 22 per cent sodium sulfate at 33°C. In the ox serum, there was an average decrease in albumin corresponding to 600 mg. of nitrogen per 100 cc., or 88 per cent of the albumin originally present. In the globulin fraction, there was

an apparent average increase of 475 mg. of nitrogen per 100 cc. It could readily be shown, however, that this apparent increase in the globulin fraction was not caused by the formation of globulin, but rather by the production of denatured albumin which, like globulin, precipitates with 22 per cent sodium sulfate. This was demonstrated by a test on some crystalline egg albumin, about 40 per cent of which was denatured by heating at 40°C. In this sample of albumin, exactly the same quantity was precipitated by sodium sulfate as by adjusting to the isoelectric point of denatured albumin. In the chicken serum, there was a decrease in the globulin fraction as well as in the albumin fraction, showing that globulin also is converted to insoluble material.

Irradiation of solutions of pure proteins showed that the changes observed in the sera were properties of the proteins as such, and not dependent on the presence of other constituents of the serum.

Samples of crystalline egg albumin recrystallized three times, egg globulin precipitated twice with ammonium sulfate, and casein dissolved in 1 per cent sodium carbonate, were irradiated. In each case, a tough, insoluble deposit was formed on the window of the cell and a small amount of non-protein nitrogen was produced. About 92 per cent of the albumin was denatured. Fifty-nine per cent of the globulin and 11 per cent of the casein separated as insoluble material. When the albumin was irradiated in 0.9 per cent salt solution instead of water, more of the denatured material remained in solution. This fact explains the apparent increase of globulin in the irradiated sera, which was noted above. Further evidence of denaturation was given by a positive nitroprusside reaction in the irradiated albumin.

It is interesting to note that the chief effect of cathode rays on proteins is similar to that of ultraviolet light observed by Harris,[4] and of sunlight as observed by Young.[5]

Although the change of greatest magnitude brought about in the proteins by means of the cathode rays was that of denaturation, there was, as stated above, both in serum and in pure protein solutions a small increase in the non-protein nitrogen. In order to ascertain whether this should be ascribed to hydrolytic cleavage of the protein molecule or to some other type of change, an attempt was made to determine the nature of the products formed.

[4] Harris, L. G., *Proc. Roy. Soc.*, Series B, 1923, **94**, 426.
[5] Young, E. G., *Proc. Roy. Soc.*, Series B, 1922, **93**, 235.

The separation of proteins from their hydrolytic products depends upon their relative solubilities in various reagents. As shown by Hiller and Van Slyke,[6] sodium tungstate precipitates a large amount of the intermediate proteolytic products, such as the proteoses, while 2½ per cent trichloracetic acid allows a much larger proportion of these to remain in solution. Unpublished experiments carried out in this laboratory indicate that 2½ per cent trichloracetic acid as recommended by Hiller and Van Slyke,[6] and even 2 per cent trichloracetic acid as used by Wasteneys and Borsook,[7] precipitate not only the original protein, but also substances formed from it which do not possess all the properties of the original material. Since it seemed likely that any hydrolytic cleavage of the protein produced by cathode rays would result in large fragments of the protein molecule, the probability existed that such products would be precipitated with the protein by trichloracetic acid. An attempt was therefore made to see if removing the protein by coagulation with heat at its isoelectric point would give

TABLE III

Comparison of Non-Protein Nitrogen in Irradiated Sera as Determined by Trichloracetic Acid Precipitation and by Heat Coagulation

Precipitation by trichloracetic acid			Heat coagulation of protein at pH 5.6		
Control, mg. per 100 cc.	Experiment, mg. per 100 cc.	Increase on irradiation, mg. per 100 cc.	Control, mg. per 100 cc.	Experiment, mg. per 100 cc.	Increase on irradiation, mg. per 100 cc.
43	58	13	43	72	29
39	50	11	59	79	20
25	36	11	45	96	51

any further evidence of the formation of such protein-like degradation products by the irradiation.

Two cc. of the irradiated serum and its control were diluted to 20 cc. with water. The pH was then adjusted to from 5.5 to 5.6, and the solutions heated to 100°C. for 2 minutes. As 5.5 is the isoelectric point of serum globulin and 5.4 that of denatured serum albumin, this procedure seemed best adapted for removing the protein by coagulation. The solutions were filtered and the filtrates analyzed for nitrogen by the Pregl[2] micro Kjeldahl method. The concentration of nitrogen in these filtrates was higher than that in the filtrates from the trichloracetic acid for both the controls and the irradiated sera, and, moreover, the difference between the irradiated and the controls was also larger (Table III). These results indicate that products are formed by irradiation which are thrown down with the protein when trichloracetic acid is used for precipitation.

These filtrates, and also the filtrates from the trichloracetic acid precipitation, were analyzed for amino nitrogen by the colorimetric method of Folin.[8] In both

[6] Hiller, A., and Van Slyke, D. D., *J. Biol. Chem.*, 1922, **53**, 253.

[7] Wasteneys, H., and Borsook, H., *J. Biol. Chem.*, 1924–25, **62**, 1.

[8] Folin, O., *J. Biol. Chem.*, 1922, **51**, 377.

cases, the filtrates from the irradiated sera contained a higher percentage of amino nitrogen than those from the corresponding controls. This difference amounted to from 1 to 3 mg. per 100 cc. It is evident, therefore, that a slight hydrolytic cleavage of the protein molecule takes place, although it is very small in comparison with the amount of protein denatured.

Tests were also made on the non-protein fraction of the irradiated sera for changes in the ammonia and urea concentrations. A slight increase in ammonia occurred, but no increase in urea. In some cases, the very small decrease in urea of 1 mg. per 100 cc. indicated that some of the ammonia might be derived from that source. When a 0.1 per cent aqueous solution of urea was irradiated for 3 hours, 11.4 per cent of the urea nitrogen was transformed to ammonia.

SUMMARY AND DISCUSSION

The effects of cathode rays on the proteins of serum appear to be (1) denaturation of a large proportion of the albumin and globulin with the formation of products that are soluble at the pH of the serum; (2) the production of a tough and exceedingly insoluble substance on the window of the cell where most of the absorption of electrons occurs; (3) a slight hydrolytic cleavage of the protein molecule producing a small quantity of products having properties so near to those of the protein that they are precipitated by trichloracetic acid but are not removed by coagulation at the isoelectric point; (4) the production of a small amount of hydrolytic products not precipitated by trichloracetic acid; and (5) the formation of a small amount of ammonia, part of which at least is derived from the urea in the serum.

It is interesting to note that these changes are such as would bring about exactly those effects on fibroblasts which were observed when cultures were grown in serum which had been subjected to cathode ray irradiation. The proteins of serum have a retarding effect on the growth of fibroblasts.[9] We might, therefore, expect their removal by denaturation and coagulation to result in the slightly larger growth which was observed. The production of SH groups in the denatured protein molecule would also tend to have a beneficial effect, as has been observed in experiments with denatured albumin. A concentration of protein split products equal to that in the irradiated sera has been observed to produce cells of characteristic appearance, full of cytoplasmic granulations and possessing long, active pseudopods, such as those noted in colonies cultivated in serum which had been subjected to cathode rays.

[9] Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1923, **37**, 653.

CELL PROLIFERATION RESPONSE TO SULFHYDRYL IN MAMMALS

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Studies made at this Institute during the past two years have brought out the fact that the sulfhydryl group is an essential stimulus to cell proliferation in simple types of plants and animals (root-tips and *Paramecium*) (1:2). The practical and theoretical bearings of this finding are clear. They have been briefly outlined in the papers cited and elsewhere (3:4). It remained to test whether the principle developed from the work with the simple forms would be demonstrable in more complex and highly developed species. Not that there was any doubt as to the essential validity of the principle, but it was felt that its exhibition in mammals would strengthen the theoretical position by extending the boundaries of support, and at the same time provide a basis for one phase of its practical application, namely, to the healing of wounds.

It was decided to combine these two aspects of the problem within a single bracket. Consequently thio-glucose was chosen as the compound to be tested. The choice was based on the fact that this substance contains the cell proliferation stimulating -SH group attached to the natural sugar glucose which presumably would provide an easily utilizable source of energy for the multiplying cells. Thio-glucose possesses other advantageous properties. Its sodium salt is fairly easily prepared in high degree of purity (5) in crystalline form. This is quite soluble in water and on neutralization with HCl yields the free -SH compound which is reasonably stable at a phosphate buffered pH of 6.8 if kept cool and in a dark place. The compound as made by Dr. Gerrit Toennies, chemist to the Institute, was used in a concentration of 1:10,000 *S*, in the experiments to be described.

Male albino rats of about 100 grams weight served as the test objects. The procedure was as follows.

The rat was anesthetized with ether or urethane and the hair removed from the back or abdomen by clipping and shaving. Two approximately circular pieces of skin, $\frac{3}{8}$ inch in diameter, were then cut out on opposite sides of the depilated area, taking care that no muscle or fascia was removed therewith.

Each wound was then covered with a small piece of gauze on which was placed a small wad of absorbent cotton. This dressing was covered with a square of thin rubber dam sealed with collodion to the body surface so that communication between the two wounds was prevented. A small hole cut in the dam over the cotton wad allowed the application of the test and control solutions to the two wounds respectively. The whole was held in place by an appropriate strip of adhesive tape encircling the body and suitably perforated over the dressings.

The test solution contained sulfur in 1:10,000 concentration as thio-glucose liberated by HCl from sodium-thio-glucose. This was buffered to a pH of 6.8 by 1.0 cc. Sorensen's phosphate mixture to 100 cc. solution. The control solution contained glucose, NaCl, and phosphate mixture in concentrations equivalent to those of the test.

Each of the two wounds was kept moist with one of the solutions by simultaneously saturating the dressing therewith every two or three hours for a period of 48 to 72 hours. At the end of this time the dressings were removed and the results recorded. Which solution contained the thio-glucose was unknown to us until this had been done, the information being solely in the hands of the chemists.

Three series of experiments were run with ten, eight, and seven rats respectively.

In the first series, eight out of the ten rats showed a distinct acceleration of wound healing on the side to which thio-glucose had been applied. In the second lot six out of the eight were similarly affected, and in the third six out of the seven. In the last two series the failures could be attributed to the fact that the rubber dam barrier between the wounds was not complete and intermingling of the solutions occurred.

The evidences of more rapid healing were as follows: More rapid contraction of the edges of the wound; lack of bleeding on removal of the gauze; obviously greater cellular growth over the surface of the wound; and in some cases inability to remove the gauze because of its interpenetration by the new growth.

The accompanying photograph demonstrates some of these points. The lower wound which had been treated with the control glucose

solution was raw and showed but little evidence of healing. The gauze though in close contact with the raw surface lifted off easily. The upper wound, to which the thio-glucose had been applied, was dry, the area was diminished, and the cells had so grown on to and within the meshes of the gauze that its removal without tissue destruction was impossible.

The advantage possessed by the sulfhydryl-treated wound was in general maintained, this coming to complete healing some days before that of the control side.

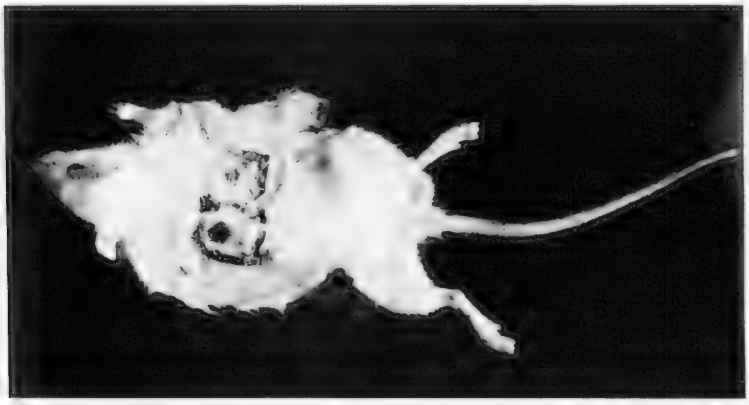

FIG. 1. Photograph showing accelerating influence of sulfhydryl on cell proliferation in the rat.

Like stimulation of cell proliferation in man has been obtained. A report of the clinical application of these findings to wound healing is being prepared by Reimann.

SUMMARY AND CONCLUSIONS

These experiments establish the fact that the sulfhydryl group is stimulative of cell proliferation in mammals as in lower organisms. The fact that the stimulation is exhibited in such a wide diversity of species including both plants and animals is justification for the belief that it is the expression of a fundamental biological phenomenon.

The literature and discussion of the subject are to be found in the papers cited.

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ANTIBODY AND AGGLUTININ IN PNEUMOCOCCUS PNEUMONIA*

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Though a number of investigators have studied the appearance of antibody and agglutinin during pneumococcus infection in man and in animals, it has seemed desirable to pursue the matter further. For this purpose, we have used blood obtained from patients with Types I, II and III pneumococcus pneumonia at the Massachusetts General Hospital and from the private practice of one of us. Through the kindness of Dr. H. A. Christian of the Peter Bent Brigham Hospital and Dr. W. H. Robey and his associates of the Boston City Hospital, we have also had the opportunity to investigate the blood of patients in these institutions. We are especially indebted to Dr. Maxwell Finland of the Boston City Hospital and to Dr. George Walker of the Peter Bent Brigham Hospital for obtaining blood from patients in these hospitals. We are indebted to Dr. Lloyd D. Felton of the Harvard Medical School for his kindness in furnishing virulent cultures of the pneumococcus and for many helpful suggestions.

Method

Tests for antibody were made by inoculating intraperitoneally each of 14 mice with 0.2 cc. of the patient's serum plus 0.3 cc. of one per cent peptone solution, and directly after this with dilutions of a highly virulent culture of pneumococcus of the same type as that found to be the cause of the pneumonia. For this purpose, seven dilutions of an eighteen hour beef heart broth culture of the organisms were made as follows:—1:100, 1:1000, 1:10,000, 1:100,000, 1:1,000,000, 1:10,000,000

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and 1:100,000,000 and 0.5 cc. of each of these dilutions injected into each of two mice.

The tests for antibody were thus done in duplicate. In addition, no serum was given to two mice used as controls, one receiving 0.5 cc. of 1:10,000,000 and the other the same amount of 1:100,000,000 dilution of the culture. All mice were observed for 96 hours. We have regarded the survival of 1 mouse of the series inoculated with the patient's serum and organisms as an indication of protection. This seems reasonable in view of the death of the control mice and the fact that in all patients whose blood was tested before treatment within the first 5 days of the illness no protection was found. We realize a possibility of error in this, but such error would change the finding in only 3 out of 57 patients.

Tests for agglutinin were made with suspensions of living or dead homologous pneumococci at varying dilutions (1:2, 1:4, 1:8, 1:16, 1:20, 1:40, 1:80, 1:100, 1:200. 1:500) of the patient's serum according to the method of Arlyle Noble (*J. Bact.*, **14**, 287, Nov., 1927), who uses small amounts of serum and concentrated suspensions of organisms. It was found that the tests were easier to read after a half hour of incubation, and this variation in the technique was made.

The blood of 57 patients was investigated for both antibody and agglutinin. On 46 of the series from two to fifteen tests were made. In the specifically treated cases the first sample of blood was taken before serum was given. Blood for subsequent tests on these cases was taken not sooner than eight hours after the administration of serum. Only 1 observation was made on 11 patients.

The results may be sketched as follows:—

Observations on Antibody in Pneumonia

Time of appearance of antibody during the disease

Our observations confirm those of other investigators in the finding that antibody in untreated cases appears at or about the time of the fall in the temperature. Of 49 patients to whom no serum had been given to the time the test was made none were found to have protection before the sixth day. Of 6 patients not treated with serum and tested at the time of the temperature fall only 1 failed to show antibody. The earliest time of appearance in the untreated group was the sixth day in 2 patients in whom the temperature fell within the next twenty-four hours.

In the treated group protective substances were frequently demonstrated after the use of Felton's antibody at a time in the course of the disease when protection would otherwise not be expected.

Behavior of Antibody during Convalescence and in Complicated Cases

Nineteen patients with antibody at the time of the fall in temperature were tested during convalescence. Protection was present in 8 from a few days to a week, in 2 into the second week, in 3 into the third, in 4 into the fourth, in 1 into the fifth and in 1 into the ninth week. In 11 of these cases no further tests were made. The remaining 8 showed that the antibody, previously present, was absent in 2 in the first, in 1 in the second, in 2 in the third, in 2 in the fifth and in 1 in the eighth week.

Four other patients who had had pneumonia 7 to 13 months previously, but had not been tested during the course of the disease, failed to show antibody.

In addition to the patients already referred to, 1 with unresolved pneumonia was found to have protection from the fifth to at least the forty-ninth day after the onset of the disease. His febrile course lasted 43 days. One patient with pneumonia and empyema was first tested 3 weeks after the estimated onset of the illness. At this time, he had antibody which persisted for at least four weeks longer. Another patient had empyema following pneumonia and had developed antibody when first tested, but died three days later of pneumococcus endocarditis, eighty-six days after the onset of the illness.

In our small series the duration of antibody after the fall in the temperature was variable. There appears to be no difference in its duration in those with and those without specific treatment. In the treated group, early or late treatment seemed to have no influence on its persistence.

The explanation of the disappearance of antibody in certain cases and its persistence in others is not clear. The presence of an unresolved pneumonia, or such complications as empyema or pneumococcus endocarditis, may have been responsible in certain cases, but in others no explanation is apparent.

Relation of Antibody to the Outcome

Of 35 patients who showed antibody at some time during the course of the disease, 29 recovered and 6 died. Of the 29 who recovered,

antibody was present at the time of the fall in the temperature in all. Of the 6 fatal cases, all were treated, none before the fifth day. One had empyema and pneumococcus endocarditis and another cardiac and renal complications. There were no obvious complications in the remaining four. All 6 had demonstrable antibody to within thirty-six hours of death.

Of 6 patients without protection during the course of the disease 5 died and 1 recovered. None were specifically treated. The patient who recovered failed to show antibody during the last three days of the disease and when tested five times during a subsequent period of twelve days.

Observations on Agglutinin in Pneumonia

The behavior of agglutinin was much the same as that of antibody. When present during the disease in patients without specific treatment it was first demonstrated at the time of the crisis or lysis. It was frequently demonstrated in specifically treated patients earlier than would otherwise be expected. It was present in all but 4 of 27 patients at the time of the fall in temperature. During the post-febrile period agglutinin was usually found to persist for a shorter period than did antibody.

Value of Tests for Agglutinin as an Indication of the Presence of Antibody during the Course of the Disease

Inasmuch as tests for agglutinin in the patient's blood are not difficult to perform the question arises whether the presence or absence of agglutinin may be useful as an indication of the presence or absence of antibody and the control of dosage of specific serum. In an attempt to answer the question, we have correlated the findings with respect to these two substances in 32 patients. Agglutinin and antibody appeared simultaneously in 8. Agglutinin preceded antibody in 5 cases, in 3 of these by one day and in 2 by two days. In 2, agglutinin was present without antibody at any time during the course of the disease. Of the remaining 17 patients, in 9 antibody preceded agglutinin by one day in 3, by two days in 1, by three days in 4 and by four days in 1. In 8 patients antibody was present without demonstrable agglutinin in repeated tests.

Agglutinin may be present even in high titer without any demonstrable antibody and conversely there may be abundant protective substances without demonstrable agglutinin. In 7 of 32 patients the presence of agglutinin if interpreted as an indication of the presence of antibody, would have led to an erroneous conclusion regarding treatment. Agglutinin, therefore, is unreliable as a control of dosage. The clinical aspects of the case seem to us a better means of judging the desirable amount of serum and the frequency with which it should be administered.

CONCLUSIONS

1. Protective substances and agglutinins were not demonstrated in the blood of patients with pneumonia untreated with serum before the fall in the temperature by crisis or lysis.

2. Protective substances and agglutinins were frequently demonstrated in serum treated patients during the course of the disease.

3. Protective substances and agglutinins were usually present at the time of the temperature fall in patients untreated with serum and persisted for days or weeks thereafter.

4. Protective substances have an important bearing on the outcome. A large proportion of those with protection recover and a large proportion without it die.

5. Agglutinin and antibody may appear simultaneously or one may precede or be present without the other.

6. Tests for agglutinin are unreliable as a control of dosage of specific serum.

ETIOLOGY OF OROYA FEVER

XVI. Verruga in the Dog and the Donkey

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Plates 21 to 23

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In the older literature on verruga[1,2,3,4] are found statements that in endemic centers the disease may sometimes occur spontaneously in domestic animals, particularly horses, mules, dogs, and pigs. That distinct differences in the appearance of the nodular lesions existed, or were thought to exist, among these animals, is apparent from the fact that they were used as criteria for describing certain types of lesions in man; for example, the deep-seated, bulky lesions were, and still are, called "mular" or "mulaire," whereas the superficial ones were sometimes referred to as "verrugas de caballo" (verruga of horses). The variation in appearance of the nodules seems to have been accepted by tradition, since careful descriptions of verruga in animals cannot be found, and actual records of experimental inoculations in the closely related species of horses, mules, and donkeys are apparently very rare. The experiment of Ribeyro, Mackehenie, and Arce,[5] in 1913, with a donkey, is the only one of the kind on record. The animal was inoculated by scarification of the skin at the inner canthus of the eye, and on the nose, with the "pulp" of a verruga nodule obtained from a patient. After 21 days the skin at the sites of inoculation showed papules 3 to 4 mm. high, which increased only very slightly in size during the next 6 days. Then they began to regress and after 11 days had entirely disappeared. The reaction seems to have been a very mild local one.

In their attempts to find an animal susceptible to *Bartonella bacilliformis*, Noguchi and Battistini[6] tried ringtail, *rhesus*, Java, and green monkeys, dogs, rabbits, mice, rats, and guinea pigs, and found that

results were definite only in the *rhesus* monkey. Later, experiments were started (Noguchi) in dogs, horses, and donkeys, the results of which are recorded below. In the dog mild verruga was induced, in the horse and in burros the results were negative so far as concerned the production of true verrugous skin lesions; in the horse and in one burro there was induration at the sites of inoculation, which lasted 3 to 4 weeks, but *Bartonella bacilliformis* was not recovered from the lesions. Further experiments, with donkeys, have been more successful.

Dog 1, female, inoculated March 23, 1926, intradermally, on eyebrows and abdomen, with (a) a mixture of 4-weeks old cultures, 1st and 2nd generations, grown on blood agar slants and leptospira medium, from the blood of *M. rhesus* 14,[7] a chimpanzee,[7] and *M. rhesus* 18,[8] and (b) a saline suspension of nodular tissue excised* from *M. rhesus* 18. 2 cc. of the culture mixture were injected intravenously. Small nodules developed after 8 days at the sites of culture inoculation, but none at the sites inoculated with nodule suspension. Four days later one of the nodules had reached a diameter of 0.5 cm. and was excised.* *Bartonella bacilliformis* was recovered in culture from a suspension of the tissue. Blood cultures made at this time were negative.

Microscopic examination of nodular tissue: In the spaces among the thick collagen fibers of the dermis there is a proliferation of endothelial cells. This process is chiefly diffuse, but small foci also are found. There is also a considerable polynuclear exudate and a moderately extensive plasma cell exudate. Except for the latter findings, the lesion is histologically similar to the lesions produced in monkeys by *Bartonella bacilliformis*.

Colt, about 3 years old. Inoculated March 30, 1926, intradermally, at two sites on the left flank, with mixed cultures (from blood agar slants and semisolid leptospira medium) of *Bartonella bacilliformis*. There was no change in the animal's temperature, which was normally 100.4°F. (38°C.), and blood culture was negative 8 days after inoculation. After 20 days there was definite induration at the sites of inoculation, which disappeared within the next two weeks. A second inoculation was made at 4 sites on the neck, on April 22, with the material inoculated on that day into Donkey 1. After 4 to 6 days there was marked induration at the two sites of culture inoculation, but none at the sites inoculated with nodular tissue and blood, and there was no fever.

Donkey 1. Inoculated April 22, 1926, at 4 sites on the left flank and 3 on the left side of the neck, with (a) 15-day culture from blood agar slant, 5th generation from M. rhesus 7,[8] (b) 14-day culture on leptospira medium, 1st generation from *M. rhesus* 29,[9] and (c) saline suspension of nodular tissue from *M. rhesus* 18,[8]

* All operations were performed under ether anesthesia.

and (d) citrated blood from *M. rhesus* 25.[8] 8 cc. of the mixture were injected into the jugular vein. There was no change in the animal's temperature, which remained at about 100°F., and blood culture was negative 4 days after inoculation. At this time there was marked edema along the neck and flank and at other points remote from the sites of inoculation. After 8 days there was definite induration at the inoculated sites, and one of the "nodules" was removed for culture and section. *Bartonella bacilliformis* was not recovered from the suspension of tissue, nor were the organisms seen in the sections. Histologically, however, the section has the appearance of that from a very early verruga lesion. Several minute foci of endothelial cell proliferation are present in the deep portions of the dermis, and there is a tendency to the formation of sheets of cells and capillaries. A moderate polynuclear infiltration is also present.

In the second donkey the inoculation gave rise to edematous areas remote from the areas of inoculation, but there was very little reaction at the inoculated sites.

Donkey 2, inoculated Nov. 30, 1926, intradermally, on the left side of the neck, and also intravenously (4 cc.), with 8-day cultures of *Bartonella bacilliformis* grown on blood agar plates. There was no change in the animal's temperature, which remained slightly below or above 38°C. Rather large, fluctuating swellings appeared within 48 hours on various parts of the body, one on the side of the back, and others on the flank and abdomen. Blood culture was negative 72 hours after inoculation, and also after 20 days. The swellings were still present 2½ months later, and one was punctured; the aspirated fluid was sterile. A piece of tissue, removed from another "nodule" at this time for section, was histologically negative. Six weeks later a saline suspension of tissue from one of the "nodules" was inoculated into *M. rhesus* 30, with negative results, both as to blood culture and local lesions. The animal's appearance was nearly normal 284 days after inoculation. Its weight at this time was 440 lbs. Reinjection of the animal 8 months later, that is, a year and a half after the original inoculation, with live cultures, produced the same reaction as before, and in this instance there were generalized small scaly eruptions all over the body, which persisted about a week.

The results in Donkey 2 were difficult to interpret, and two more donkeys were inoculated, one with killed cultures of *Bartonella bacilliformis*, and the other with a filtrate of living cultures. The reaction in these two animals was much the same as in Donkey 2. In the animal receiving the filtrate (Donkey 3) the swellings appeared within 72 hours after inoculation (Fig. 1); in Donkey 4, inoculated with heat-killed cultures, they did not develop fully until about 10 days after inoculation. In both, however, they persisted for several weeks.

The study of sections made from the swollen areas yielded no explanation of the phenomenon which occurred in these two animals.

Donkey 3, weight about 500 lbs., injected April 4, 1928, intravenously, with filtrate (Berkefeld V) of 14-day cultures of *B. bacilliformis*. The temperature was 102.4°F. late in the day of inoculation, but thereafter remained a few tenths of a degree below or above 100°. Large raised areas developed in 72 hours, as in Donkey 2 (Fig. 1). Blood culture was negative 9 days after inoculation. Control *rhesus* monkeys (injected with the same material, intravenously and intradermally) developed no skin lesions, and blood cultures were negative. Cultures of the inoculated material also proved its sterility.

Donkey 4, 650 lbs., injected April 16, 1928, intravenously, with cultures of *Bartonella bacilliformis* killed by heating 30 minutes at 60°C. This material was proved sterile by culture tests and animal inoculation. The animal's hide was not perfectly smooth, and the natural prominences were outlined with a skin pencil before inoculation. 10 days after inoculation this animal was found to have developed the same swellings as Donkeys 2 and 3, but they were less marked. The appearance of the animal 25 days after inoculation was similar to that of Donkey 3. A piece of tissue removed from a swelling on the left side of the animal 8 months after inoculation was found on microscopic examination to consist solely of fat tissue.

The donkeys used in the first four experiments were of the small (440–650 lbs.) light gray variety commonly known as the "burro." The fifth donkey, in which definite verruga lesions were produced (Fig. 3), was a larger animal (800 lbs.) and brown in color. So far as can be ascertained, however, the species is the same.

Donkey 5. Weight 800 lbs. Five intradermal injections were made, on Jan. 5, 1929, with a mixture of living cultures of the 5 strains of *Bartonella bacilliformis* on hand in the laboratory,[10,11] two into the skin of the left flank, two on the right ear, and one on the left ear. 10 cc. of the same mixture were injected intravenously. The material produced, on injection into the skin of the ears, swellings about 1 cm. in diameter, which did not subside but slowly increased in size, so that after 6 days they were 2 cm. in diameter and 12 mm. high (Fig. 3). They were dome-shaped and slightly fluctuant. The skin over them remained intact and was of the normal black color of the donkey's ear. The sites of inoculation on the flank secreted a clear serous liquid for a few days, without papule or nodule formation, and then completely healed.

The animal continued to receive intravenous injections of mixed strains of living cultures of *Bartonella bacilliformis*, 20–30 cc. at each dose, twice a week for 14 weeks, the idea being to produce, if possible, a strong local and general reaction, in the hope of obtaining an immune serum for use in other experiments.

After the nodules on the ears had reached a diameter of 2 cm. they remained stationary for a period of 3 weeks, after which they gradually became smaller until at 72 days after inoculation they were invisible. Blood cultures were made repeatedly throughout the period of observation and yielded uniformly negative results.

One of the nodules on the right ear was aspirated on the 22nd day after inoculation and slightly less than 1 cc. of turbid liquid obtained which proved by culture tests to be sterile. Both nodules on the right ear were punctured three days later, but no liquid was obtained from either. The nodule on the left ear, similar in all respects to those on the right ear, was then removed under novocaine anesthesia. A portion was used for inoculation of a *rhesus* monkey, and another portion for histological study.

Animal passage. *M. rhesus* D-1 was inoculated intradermally and by scarification on Feb. 16, 1929, with a saline suspension of a part of the nodule removed from Donkey 5. After an incubation period of 9 days typical nodules (Fig. 4) developed at the sites of inoculation, and blood culture made the following day was positive for *Bartonella bacilliformis*. Nineteen days after inoculation the nodules were mature, the area inoculated by scarification showed fully developed lesions, and after 51 days recovery was complete. The disease ran a typical course. The culture of *B. bacilliformis* obtained from the blood of this animal and a subculture from it, on intradermal inoculation into *M. rhesus* D-2 and D-3 produced typical reactions (Figs. 5 and 6).

Histological examination (*Figs. 7 and 8*). The tissue removed from the ear of Donkey 5 consisted of the covering epidermis, a narrow rim of normal skin encircling the nodule, and a superficial portion of the nodule itself. The latter, irregularly marked off from the rest of the tissue, was soft, gray with a faint pink tinge, and about 4 mm. thick at the center, tapering off gradually in thickness toward the edges. The tissue was fixed in Regaud's fluid, and sections were stained with hematoxylin and eosin and with Giemsa's solution.

The hematoxylin and eosin sections, examined with the naked eye, show a thin normal layer of epidermis covering a layer 1 to 1.5 mm. thick of apparently normal dermis. Beneath this is a collection of small round bodies, closely packed and tending here and there to become conglomerate. Each of these individual nodules, varying from 1 to 3 mm. in diameter, stands out from the rest of the tissue by its deeper hematoxylin stain. Together they comprise the nodular tissue proper.

Microscopically the minute nodules which make up the large nodule are in general fairly distinct from one another, but in places they tend to fuse. They are all very densely infiltrated with polynuclear leucocytes, which somewhat obscure the underlying structure by their presence. However, numerous slit-like spaces of variable length are seen within the minute nodules, lined with large, oval, flat cells with clear oval nuclei. These cells resemble endothelial cells. Some of the spaces contain serum; others are empty. With higher magnification

there are seen larger collections of these endothelial cells forming sheets or running in bands, and having a tendency to form blood-containing capillary-like structures.

The overlying epidermis and the narrow zone of dermis, with its numerous hair follicles and sweat glands, are normal. Though there is no distinct capsule, these tissues are separated sharply from the collection of minute nodules beneath. The surgical line of excision passes through the nodular material.

These findings show that the unit of the pathological process situated in the deep portions of the dermis is a minute nodule, from 1 to 3 mm. in diameter. The units may be discrete or tend to fuse. Each is composed of a considerable proliferation of endothelial cells, arranged in sheets, bands, and tubes. Superimposed, there is a very marked polynuclear infiltration, as was found in the section of nodular tissue from Dog 1.

SUMMARY AND CONCLUSIONS

In the experiments here reported, definite verruga lesions, in which the presence of *Bartonella bacilliformis* was established by culture or by passage to *rhesus* monkeys, were produced in a dog and in a donkey by inoculation of cultures or monkey passage strains. The reaction induced in these animals was entirely local, however; blood cultures were sterile. Histologically, the lesions produced were similar to those obtained in monkeys by inoculation of *Bartonella bacilliformis*, except for the presence of a marked polynuclear leucocytic exudate.

In another donkey a lesion histologically suggestive of verruga was produced, while in one donkey and a horse the results of inoculation were negative or indefinite.

The intravenous injection of a filtrate or of heat-killed cultures of *Bartonella bacilliformis* into two donkeys was followed by the appearance of large, soft, subcutaneous swellings, on various parts of the body, not resembling in any way verruga lesions.

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EXPLANATION OF PLATES

PLATE 21

FIG. 1. Donkey 3, 72 hours after intravenous injection with a filtrate of cultures of *Bartonella bacilliformis*, showing the large swellings which arose on the side.

FIG. 2. Photograph of the same animal, taken before inoculation. For comparison with Fig. 1.

PLATE 22

FIG. 3. Appearance of nodules on right ear of Donkey 5, 39 days after inoculation. The nodule on the left ear had been partly excised 14 days previously, and the wound was in process of healing.

FIG. 4. *M. rhesus* D-1, showing verruga nodules on abdominal wall 10 days after inoculation with emulsion of nodule from ear of Donkey 5.

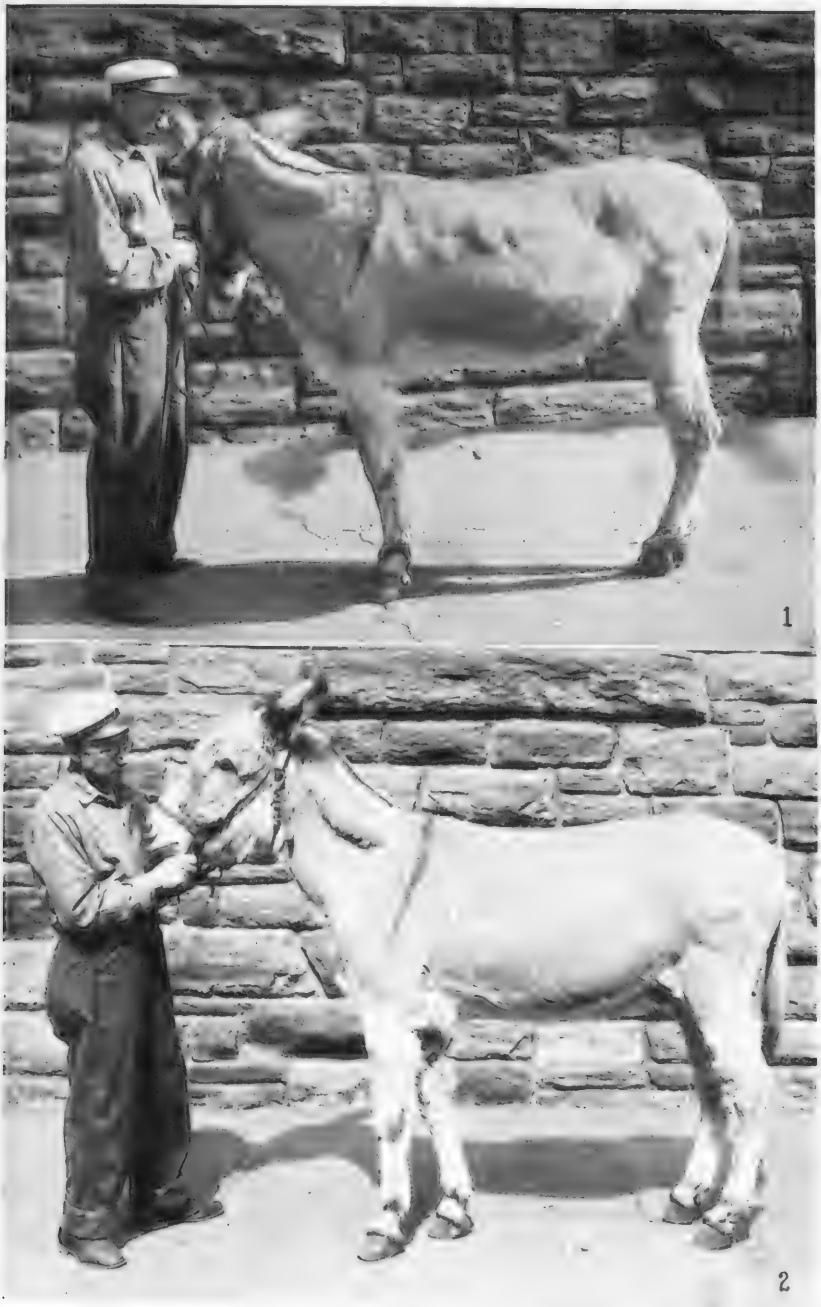
FIG. 5. *M. rhesus* D-2, 31 days after inoculation with culture of *Bartonella bacilliformis* from blood of *M. rhesus* D-1.

FIG. 6. *M. rhesus* D-3, 22 days after inoculation with subculture of *Bartonella bacilliformis* from blood of *M. rhesus* D-1.

PLATE 23

FIG. 7. Low power magnification (× 105) of section from nodule on ear of Donkey 5, showing one unit of the lesion.

FIG. 8. Higher magnification (× 600) of section from same nodule, showing proliferation of endothelial cells, lymph and blood spaces, and polynuclear infiltration.



(Noguchi *et al.*: Etiology of Oroya fever. XVI)

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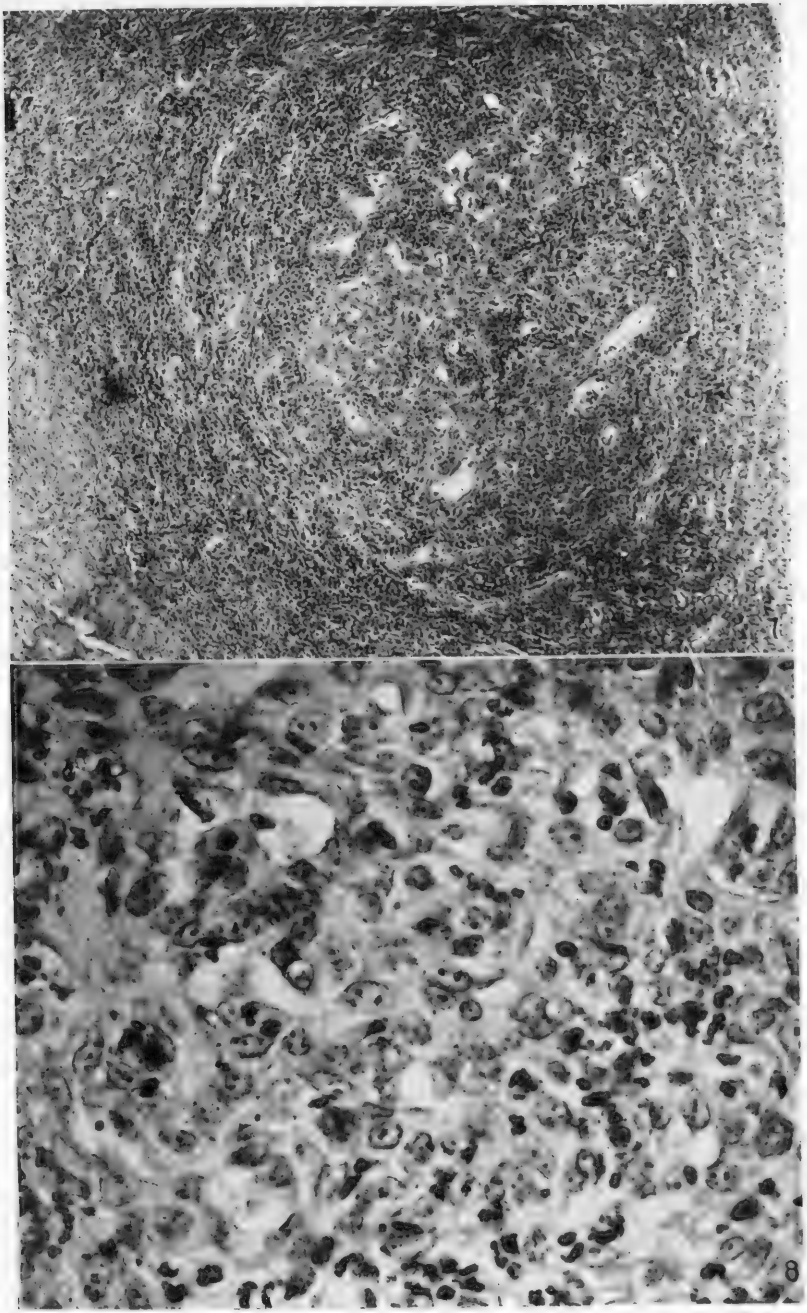

Photographed by Louis Schmidt

(Noguchi *et al.*: Etiology of Oroya fever. XVI)

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DIFFERENTIATION OF HEMOLYTIC STREPTOCOCCI OF HUMAN AND OF DAIRY ORIGIN BY METHYLENE BLUE TOLERANCE AND FINAL ACIDITY

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Although hemolysis on the blood agar plate was formerly considered a property peculiar to streptococci from pathogenic sources, it is now recognized that the same property is also possessed by many non-pathogenic strains. Means of distinguishing hemolytic strains that are pathogenic from those that are non-pathogenic is especially important in the sanitary examination of dairy products. Previous studies (1–9) on streptococci have dealt with the properties of hemolysis, final acidity, and reduction of dyes, but the correlation of the results has been made difficult by the fact that the different workers have had different interests. The medical bacteriologist has usually studied strains isolated from infections and has emphasized hemolysis or acid production without regard to dye reduction. The agricultural bacteriologist on the other hand has studied non-pathogenic or saprophytic strains isolated from milk products and has emphasized acid production or dye reduction without regard to hemolysis.

The present investigation was planned to attempt to correlate these differential characters previously used in their respective fields by medical and agricultural bacteriologists. In spite of the fact that no new methods were introduced, the results are of new interest: (1) the collection of hemolytic streptococci included both pathogenic strains representative of those associated with human disease and saprophytic strains representative of the normal flora of milk products; (2) the three tests (hemolysis, final acidity, and dye reduction) are applied at the same time to strains from the different sources.

EXPERIMENTAL

Final Acidity and Source of Strains. The 138 strains of hemolytic streptococci included in our tests may be separated on the basis of their final hydrogen ion concentration into the two main groups described by Ayers (1) and by Avery and Cullen (2): those having a final acidity range of pH 5.3 to 5.0 represent the low acid group; the others with a range of pH 4.5 to 4.0, the high acid group.

TABLE I

Hemolytic Streptococci

Source	Number of strains
Low acid producing strains	
Sputum, pneumonia	6
Blood, septicemia, meningitis, osteomyelitis	7
Pleural fluid, empyema	19
Lung, pneumonia	12
Throats, pneumonia, measles	43
Pus, abscesses, cellulitis, scarlet fever	4
Cows' udders	4
Total	95
High acid producing strains	
Cheese	21
Butter starter	1
Milk (pasteurized 1, certified 2)	6
Udders	14
Cow feces	1
Total	43

Table I summarizes the grouping of the strains on the basis of final acidity of glucose broth cultures. All the strains from human sources fall in the group of low acid producers. While 4 of the strains included in the low acid producing group were isolated from the udders of cows, two of them were identified as belonging to the human type and were known to be responsible for two separate epidemics of septic sore throat. Hence, all but two of the 95 strains in the low acid

producing group were definitely associated with human infection. In contrast to this, all of the 43 strains producing high acidity had been isolated either from bovine sources or from dairy products.

Methylene Blue Reduction

All strains both of the low and high acid producing groups were tested for their capacity to reduce methylene blue in milk.

Three different concentrations of the dye were used: 1 to 20,000, 1 to 10,000 and 1 to 5,000. Methylene blue milk was prepared by adding a sterile 0.4 per cent aqueous solution of medicinal methylene blue (Merck's) to sterile fat-free milk in amounts sufficient to give the desired final concentration. Three tubes of methylene blue milk, containing respectively 1 to 20,000, 1 to 10,000 and 1 to 5,000 concentrations of the dye, were inoculated with 0.1 cc. of 24 hour broth culture of

TABLE II

Reduction of 1: 5000 Concentration of Methylene Blue in Milk by High and Low Acid Producing Strains

Final acidity	Methylene Blue Reduction	
	Strains not reducing	Strains reducing
Low acid producing group	94	1
High acid producing group	19	24

the strain to be tested. The inoculated tubes were incubated at 37°C. and observed for dye reduction over a period of seven days.

The tests with 1 to 5,000 concentration of methylene blue furnished the most definite distinction. As shown in the summary (Table II), 94 of the 95 low acid producing strains failed to reduce methylene blue. (Ninety-one of these low acid producing and non-dye reducing strains were isolated from human source). On the other hand, the high acid producing strains, all of which were isolated from dairy sources, can be separated into two groups; strains which reduced and those which did not reduce the dye. It is important that 14 of the 19 high acid producing strains that failed to reduce the methylene blue had been isolated from udders of cows; and that 21 of the 24 high acid producing strains that reduced the dye were from various kinds of cheese.

Bactericidal Action of Methylene Blue

Negative subcultures were obtained in tests of viability of the streptococci introduced as the test inoculum in the cultures in which no reduction of the dye had occurred. The apparent absence of living streptococci indicated that failure to reduce the dye in the preceding tests represented a lack of tolerance to methylene blue on the part of the non-reducing strains. Apparently in the unreduced state, the methylene blue either prevented the growth of the non-reducing strains (bacteriostatic action) or destroyed their life (bactericidal action).

In order to obtain further information on the influence of methylene blue upon the viability of the streptococci a constant amount (0.1 cc.) of broth cultures of reducing and of non-reducing strains was inoculated into milk containing a known concentration of the dye; plate cultures were made after different intervals of time. The original number of streptococci added to the methylene blue milk was known to be quantitatively sufficient to establish the growth of the more delicately growing strains in the medium containing no methylene blue.

The results of the typical experiments summarized in Table III show that approximately 90 to 97 per cent of the non-reducing streptococci were killed by 10 minutes exposure to a 1:5000 concentration of methylene blue in milk. Different strains of the non-reducing group varied somewhat in the time required to kill 100 per cent of the cells; with some strains, complete sterility was obtained within 30 to 60 minutes but with others a few viable cells persisted for several hours; in all cases, the cultures were completely sterile within 48 hours.

DISCUSSION

A collection of hemolytic streptococci from both human and dairy sources was studied in order to determine if the combined use of the properties of dye reduction and of final acidity would be of value in the differentiation of hemolytic strains of diverse origins.

Different concentrations of methylene blue were tried but it was found that a solution of 1 to 5,000 in milk gave the most clear cut separation of the strains into dye-sensitive and dye-tolerant groups. This concentration of the dye not only prevented growth but was

actually bactericidal to the dye-sensitive strains. The dye-tolerant strains grew in the presence of and reduced a 1:5000 concentration of methylene blue. Of the 113 dye-sensitive strains, 108 were isolated from human or bovine sources and in many instances were associated with infectious processes in man or cattle. The other five strains were from samples of fresh and certified milk and were probably of udder origin (hemolytic udder streptococci are more commonly found in freshly drawn and certified milk than in ordinary market milk where the more resistant varieties of milk streptococci predominate (7, 9)). In contrast to the direct animal origin of the dye-sensitive hemolytic

TABLE III

Hemolytic Streptococci; Bactericidal Action of 1:5000 Methylene Blue Milk

Period of Exposure to Methylene Blue 1:5,000	Number of viable organisms per cmm.			
	Strains not reducing Methylene Blue		Strains reducing Methylene Blue	
	Strain "2"	Strain "4"	Strain "P"	Strain "M"
minutes				
0	2,200	3,500	2,500	3,000
5	460	770	2,400	2,700
10	40	320	2,000	2,800
15	9	300	3,000	3,600
20	1	210	4,500	6,000
30	0	60	6,000	7,100
60	0	25	11,000	13,000

streptococci, the 25 dye-tolerant strains with only two exceptions[1] were isolated from pasteurized milk, cheese and commercial butter starter.

When both the methylene blue and the final acidity tests are applied to hemolytic streptococci from human and dairy sources, three groups are distinguished. First, a group of low-acid-producing strains that are sensitive to methylene blue; this corresponds to Avery and Cullen's low-acid-group from human sources. Second, a group of high-acid-

[1] Although one of these strains was isolated from a human throat and the other from a cow's udder, their presence in these locations might have been due to their introduction from saprophytic sources; e.g., recently ingested food in the case of the throat strain, or the cow's bedding in the case of the udder strain.

producing strains that are sensitive to methylene blue, these are most commonly derived from the normal or diseased udder. Third, a group of high-acid-producing strains that are tolerant to methylene blue. This last group, the dye-tolerant and high-acid-producing hemolytic streptococci are different from the high-acid-producing but dye-sensitive strains included in Avery and Cullen's group of "bovine" strains. The latter are most frequently of udder origin and are often associated with bovine mastitis; their presence in dairy products is for the most part limited to fresh raw milk for they are relatively infrequent in older market milk or in stored dairy products like butter or cheese. The dye-tolerant group on the other hand are in general much more resistant to unfavorable conditions than are most hemolytic streptococci and consequently are found in cheese or other dairy products that have been subjected to storage or other processes which tend to kill off the dye-sensitive strains.

The grouping of streptococci upon this basis, while not an attempt at a systematic classification of the *Streptococcus* genus, is of interest from two different points of view. The results as a whole indicate, that with hemolytic streptococci there is an apparent relation between lack of tolerance to methylene blue and tendency toward parasitism; and that all three tests, namely hemolysis, final pH, and methylene blue reduction should be applied in the study of streptococci of disputed etiological and epidemiological significance.

SUMMARY

A grouping of 138 strains of hemolytic streptococci based on differences in dye-sensitiveness and in final hydrogen-ion concentration of cultures is presented. Three groups are distinguished; (1) human parasitic strains, defined by a final pH range of 5.2 to 5.0 and by failure to reduce methylene blue (1:5000) in milk, (2) bovine strains parasitic in the udder, characterized by a final pH range of 4.5 to 4.2 and by failure to reduce methylene blue (1:5000) in milk, (3) saprophytic strains, characterized by a final pH range of 4.5 to 4.2 and by ability to reduce methylene blue.

Methylene blue was bactericidal for the strains of hemolytic streptococci that fail to reduce it, but neither bacteriostatic nor bactericidal for the strains that caused its reduction.

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STUDIES OF TISSUE MAINTENANCE

III. Persisting Bloodlessness after Functional Ischemia

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When the circulation reenters a part that has been briefly deprived of blood there ordinarily develops in it an active hyperemia, as every student of vascular physiology knows. But if the deprivation has endured for a somewhat longer time the happening is diametrically different. As the present paper will show, an obdurate local vasoconstriction has in some way been invoked, one so effective that the tissue involved remains closed off from the circulation for a greater or less period even though the flow through neighboring regions of the same sort is unusually good and the systemic blood pressure above the original level.

Persisting ischemia of the sort here described can readily be demonstrated with the aid of highly diffusible vital dyes, in animals with blood bulk reduced by bleeding or by solutions inducing anhydremia. Under such circumstances a patchy ischemia of the superficial tissues develops after some minutes, one so complete that the patches become pronouncedly acidotic, and even highly diffusible vital stains (phenol red, brom phenol blue, Patent Blue V) fail to enter them (1). If the blood bulk be restored soon after these patches have developed they rapidly disappear; but when they have endured for a little while the restoration has merely the effect of accentuating them through the passage of additional stain into their surroundings, and an hour or more may elapse before, by a gradual coloration, they are lost in the general hue.

Experiment 1.—With barium sulphide the hair was carefully removed from the body and thighs of a well nourished and vigorous, white female cat. No cutaneous inflammation ensued during the next few days. The animal was kept in a well-warmed room. For twenty-four hours prior to operation it was given no food,

but was allowed water. Under ether the trachea was cannulated with a Y tube for the purposes of anesthesia; the right carotid was connected with a mercury manometer for blood pressure readings; and the left axillary artery and a vein of the left foreleg (radial branch of the cubital) were cannulated, for bleeding and the injection of solutions respectively. All this took 9 minutes. The cat was then laid upon its back on an electrically warmed pad. The limbs assumed natural positions. The carotid blood pressure was constant at 142 mm. Hg.

Now 30 cc. of blood was taken from the axillary artery in the course of 3 minutes 15 seconds, and 16 minutes later 17 cc. more (in 3 minutes), and after the lapse of another 10½ minutes 8 cc. more (in 2 minutes). In this way the carotid blood pressure was reduced to 74 mm. 31 minutes from the beginning of the depletion an injection of phenol red was made. The cat weighed 2240 gm. and the warmed 4 per cent dye solution (at pH 7.4 and isotonic with the blood) was given in the ordinary proportion of 3½ cc. per kilo, 8.25 cc. being gradually run into the vein during 80 seconds. After the injection the pressure did not improve, but tended to fall as in other animals bled to the limit and injected only with fluid which would not remain in the vessels. Three minutes later, when the pressure was only 60 but the breathing good, the lips, gums, and conjuctivae had become deep red, with an orange cast suggestive of acidosis, whereas the trunk and thighs showed merely a few orange patches widely separated by pallid unstained regions. By this time a normal cat would have been brilliantly red.

The blood, as taken, had been defibrinated with care so that as little as possible should be lost. It was now aerated, warmed and restored to the animal by successive injections. A mere 4½ cc., given 3 minutes after the dye was all in, brought the pressure to 110 millimeters and 15 cc. 10 minutes later carried it to 150, that is to say above the initial level. Despite this recovery the staining scarcely progressed; and two further injections of all the remaining blood (22 cc. in 1 minute 45 seconds and 9½ cc. in 35 seconds) were made 18 and 20 minutes respectively after the dye injection. The carotid pressure reached 160 and remained at that figure for the next three quarters of an hour. The heart beat violently and the arterial pulsations were accentuated. After the return of the blood the deeply stained conjunctivae, gums and lips practically at once became red without trace of orange—between *jasper red* and *eugenia red* (Ridgway) (2)—showing that the slight general acidosis had disappeared. Over the trunk and thighs, however, the changes were very gradual. Large areas were still unstained 3 minutes after the final blood injection, and where the dye penetrated it had a hue between *ochraceous orange* and *ochraceous buff*. Little by little the coloration extended, but even after half an hour there were still many unstained patches such as are ordinarily associated with a diminished blood bulk (3). The areas to which the phthalein had originally been distributed were now turning red whereas those into which it penetrated later were orange. These various local differences caused the body surface to appear piebald with orange, red and white. Forty-six minutes after the last return of blood there were no longer any unstained areas but a pronounced

spotting of pale orange upon red persisted. The orange patches were now small, only ½ to 1 cm. in diameter.

To hasten the disappearance of the patches 40 cc. of warm Ringer's solution was injected intravenously 47 minutes after the last return of blood. The injection took 2 minutes. The blood pressure, which had been at 158, fell transiently but recovered to 152. Within the next twenty minutes all the orange spots disappeared.

In this experiment the patchy ischemia induced by the bleedings lasted at least half an hour after the blood had been restored and very probably for more than an hour, if one can judge from the persistence of areas of local acidosis that had been slowly colored by diffusion inwards from the well-stained tissue round about. The preliminary bleedings had been done gradually and at intervals, in order to allow opportunity for readjustments within the organism, and more especially for the passage of fluid out of the tissues into the vessels to make up the blood bulk. As Starling has shown (4), compensatory changes of the latter sort are practically at an end within 5 minutes after a single brisk bleeding, and subsequent bleedings do not elicit them in any important degree. The depletion was pushed very nearly to the limit, as indicated by the tendency of the blood pressure to fall after it had been accomplished, despite the introduction of dye dissolved in a salt solution. The inability of salt solutions to restore the blood pressure on such extreme occasions is well recognized. Only 51 cc. of blood was returned as compared with 54 cc. taken, but the said 54 cc., as drawn from the vessels, consisted in part of tissue fluid; for, as is well known, the last blood removed at even a rapid bleeding is dilute as compared with the first. It follows that the actual quantity of blood taken was less than our figure would indicate. The circulatory state after the reinjection supports this view. It was one of evident plethora, the heart beating violently, the pulsation of the arteries unusually prominent, and the veins engorged.

The quantity of phenol red injected into the depleted animal was not reduced from that which would have stained a normal cat evenly and deeply. The blood bulk, though, had been reduced by nearly half at the time of the dye injection. The question comes up of whether under such conditions the persistent patching may not have been caused by an untoward influence of the dye. True, previous experi-

ment had repeatedly shown that an acidotic patching indicative of local ischemia develops when the vital staining precedes the bleeding (5), which last removes much of the dye still in circulation. But this does not mean that the patches would have persisted had the blood been restored to the body. To determine what influence, if any, the dye had in the persistence of the patching was not difficult.

Experiment 2.—A vigorous, male, white cat of 2240 gm. was prepared like that of Experiment 1, save that it was fasted for only 16 hours prior to operation. The same cannulations were performed, under ether, in 19 minutes. The carotid blood pressure was found to vary between 140 and 145 mm. of mercury. A bleeding of 38 cc. in 120 seconds was now carried out, and others followed of 12 cc. in 70 seconds, 15½ cc. in 90 seconds and 6 cc. in 45 seconds, 14½, 23½ and 51½ minutes respectively from the beginning of the first. The carotid pressure was in this way brought down gradually to 84 mm. Hg. The body surface had become notably pallid, with suggestions of even paler blotches here and there. The animal was still in excellent condition, stirring under the light ether. The blood pressure tended to fall after the last bleeding. It decreased to 74 mm. Hg in a period of 9 minutes, when the reinjection of blood was begun, the animal being in good condition at this time. Less than 2 cc. had been lost in defibrination but 8.4 cc. more was withheld, since the intention was to throw that much phenol red solution into the circulation, and the avoidance of a sudden plethora seemed desirable. All the rest of the blood, aerated and warmed, was introduced by way of a foreleg vein in the course of three minutes, and was followed immediately by the 4 per cent phenol red solution (during 65 seconds). The blood pressure rose to 148 and fell again slowly to 130. The tip of the nose and the gums became red but the superficies of the trunk and thighs colored very slowly, and where the phthalein entered it was at first orange, not red. Large patches remained wholly uncolored. 5 minutes after the dye injection the regions between these patches had for the most part changed from orange toward red, showing that the local acidosis was being dissipated; and the dye was slowly extending into the margins of the uncolored regions as a narrow zone of orange. After another half minute the remaining 8.4 cc. of blood was returned to the body. The pressure rose to 158 and soon to 168, near which high level it long remained. The coloration continued strikingly various. 7 minutes after the final injection of blood there were still many irregularly distributed, uncolored patches 1½ to 2½ cm. in diameter, of serpiginous outline. They were scattered thickly upon a background of varied hues, from *carrot red* to *old rose*, and at their periphery one could see a narrow zone, or edging, of *ochraceous buff*. The marked increase in the blood pressure facilitated the passage of the stain into the tissues where circulation was taking place, with result that 11 min. after the last blood injection the patches were even more pronounced than before because of the deeper red of the background. The mucous membranes of the mouth were now *eugenia red*, and the pads of the paws pink,

evidence that there was no blood acidosis; but the hue of the stained superficial tissues had some orange in it here and there. After 14 minutes many of the spots were disappearing; and the general hue was *light jasper red*. After 18 minutes stain had entered all the remaining patches, coloring them an orange buff. The blood pressure had now fallen to its initial level, 140 mm. Hg. Some of the orange spots were still to be discerned 55 minutes after the last injection of blood; but they had gone after another 34 minutes, when the surface color was everywhere the same, a hue between *eugenia red* and *old rose*. The blood pressure meantime had varied between 140 and 166. The ether was purposely kept very light.

These observations in which phthalein was injected after the blood had again been returned make plain the fact that the obdurate ischemia cannot be due to an influence of the vital dye. It is no artefact. That the bloodless state existed prior to the injection of phthalein was clear, not alone from the failure of the latter to enter large areas here and there, but from the hue it took on at the edges of these areas, a hue indicative of preexisting local acidosis such as develops only when ischemia has endured some time (6). There were vague indications of a special patching of the pallid skin even before the return of the blood. The intensification of the patches after the final blood injection, which resulted from a deeper staining of the tissue between them, is no unique phenomenon. We have regularly observed it, and,—as one would expect,—even more pronouncedly, when the blood that was withdrawn and returned to the body contained dye.

Prior to a consideration of the causes for the persistent bloodless state the circumstances of its occurrence will be more nearly dealt with. Those leading to the initial patchy ischemia have been described in several previous papers (7). The patches develop in compensation for a reduced blood bulk, however brought about; and they appear only when the reduction has been considerable. The greater the depletion, the larger are the bloodless areas; and if it persists unrelieved they increase in size by peripheral extension. In animals gradually depleted by induced anhydremia they appear before any noteworthy reduction of the general blood pressure occurs; but in hemorrhage cases, they are best seen when the blood pressure has been substantially lowered. They occur in both urethanized and etherized animals but develop also in those bled under local anesthesia, a relatively great depletion being required to cause them in these last. They occur when the skin is kept warm artificially as well as when it has grown cold owing to deficient blood flow.

The persistence of the patching after return of the blood to the body is dependent, like its initial occurrence, on the degree of the depletion and its duration. The more the blood bulk is diminished, and the longer the elapsed period afterwards, the more obdurate does the patching prove.

Experiment 3.—A thin, white, female cat, with a few gray markings, previously depleted and fasted for 24 hours, was operated upon under ether to cannulate the trachea, right carotid artery and left jugular vein. The animal weighed 1950 gm. The operation took 15 minutes. The initial blood pressure was irregular varying from 120 mm. Hg to 150 mm. With the animal on its left side in an unconstrained position 56 cc. of blood was removed at three bleedings, by way of the cannulated carotid, in the course of 40½ minutes, reducing the blood pressure to 38 mm. at which low level it remained, until,—after 45 minutes in all,—7½ cc. of phenol red solution was injected, in 100 seconds. The animal appeared *in extremis* after 47½ minutes; and 2½ cc. of the heparinized blood was reinjected. Artificial respiration was necessary for a few breaths. It was given with the Gates' pump (8). Then the pressure rose to 50 mm. and natural breathing was resumed. After a further 2 minutes 3 cc. more of blood was injected, merely sufficient that is to say to maintain the animal. The vital staining was for some time nearly negligible except that the lips and gums, which had appeared bloodless, promptly became red after the injection of the phthalein; but little by little color crept in, and 12 minutes after the last blood injection (or 15 minutes after the phthalein) the surface was strikingly variegated with orange to red areas betwixt large unstained patches. The pressure had reached 60 mm. Hg. Now 3 cc. more of blood was given, and 7½ minutes later all the rest (in the course of 4½ minutes) except 9 cc. The pressure at once rose to 152 millimeters and the veins became notably distended, yet four minutes later the mottling had not altered. Thereafter the red between the patches became accentuated and these slowly turned orange with dye entering them, and eventually they merged with the red of their surroundings. The last of them was not gone for about an hour. To the blood as it was withdrawn salt solution, containing 10 mg. of heparin (Hynson, Westcott and Dunning) in every cc. was added in the proportion of 1 cc. for 15 cc. of blood. What with this addition, the amount of fluid returned to the body, though 9 cc. less than the total of blood plus heparin, was only 5 cc. less than the total of blood removed. A large experience with the anticoagulant enables us to say that it does not of itself induce local ischemia.

In this experiment the animal was bled to the limit and the blood was only very gradually returned to the body after the injection of phenol red. At first the dye largely failed to enter the superficial tissue, and where it did so later the color indicated tissue acidosis. Needless to say the very low blood pressure will amply account for these find-

ings. Later on, when the unstained patches were smaller and well demarcated, all of the blood except 5 cc. was gradually returned to the body. Although the blood pressure mounted far higher than before the bleedings, and there was a manifest plethora with engorgement of the veins, the spotting persisted for an hour. These results and those of Exp. 1 may be contrasted with the following:—

Experiment 4.—A well nourished, white female cat of 2540 gm. was prepared and operated upon as in the case of Exp. 1, except that the removal of fur had been done with the razor and the initial fast had lasted only 18 hrs. The cannulations, under ether, took 18 minutes. Thereafter the animal was bled 62 cc. in 54 minutes. The blood pressure was reduced from 170 mm. Hg merely to 100 mm. Pallid blotches on a generally pale skin became visible over the animal's right side—which was uppermost; and 19½ minutes after the last bleeding 9½ cc. of phenol red was injected (in 65 seconds). 4 minutes later the body surface was a brilliant pink variegated with numerous, scattered white patches up to 2 cm. in diameter. The return to the body of all the defibrinated blood was begun 11 minutes after the dye injection and completed 3½ minutes later. The carotid pressure rose to 180, at which level it remained, and there was the usual evidence of a plethora. Within a minute the animal had stained much more deeply, and the spots stood out in startling contrast; but they soon began to turn pink and within thirteen minutes had disappeared. The cat was by now of a hue deeper than *old rose*.

In this instance although much blood had been taken the carotid pressure remained fairly high and the blood was returned to the body after only a short interval. The ischemic patches, while well defined, had not endured long, for no acidosis had developed in them. They stained pink, not orange, and quite rapidly.

To these contrasted findings may be added others of similar import in rabbits. Very deep urethane anesthesia so impairs the circulation that patching results, but ether does not do this unless pushed close to the lethal limit (9). In the tests now to be described the animals were bled with the aid of a local anesthetic, novocaine.

Experiment 5.—A white, female rabbit of 2400 gm. shaved over the trunk and thighs 9 days previously was fasted 24 hours, and under ether the right common carotid was brought to the surface and cannulated. The vessel was kept shut by a special device made of rubber tubing (10), the pressure of which could be relaxed at will and bleeding accomplished with the animal sitting naturally. The incision was swabbed with novocaine and covered with a wet dressing. When the animal was again up and about, after 63 minutes, novocaine was re-applied

locally and the depletion begun. During the course of an hour and five minutes 76 cc. of blood was removed—at three bleedings—and at once defibrinated. Immediately afterwards the animal appeared in good condition, sitting in the normal posture; but it was found collapsed and apparently moribund 18 minutes later. The reinjection of all of the blood by way of an ear vein, and removal to a very warm room, benefitted it but little. Eight minutes later, at the time when 9 cc. of phenol red was given, it was still pallid and appeared unconscious, but after another eleven minutes it got to its feet. The superficial staining at this time was localized to localities where large vessels entered the skin (popliteal space, regions just back of the axilla and over the sacroiliac joint). Here the hue was orange. The animal was sitting up and in fair condition 51 minutes after return of the blood, yet the skin still showed completely unstained areas on a ruddy to orange ground. Along the ridge of the back was a well demarcated orange stripe. The general condition continued to improve, but 79 minutes after the blood injection the patches previously unstained were still to be discriminated, though now *coral red* on a *jasper red* background. At this time 7.2 cc. of warmed 4 per cent brom phenol blue was injected in 60 seconds. Most of the surface of the animal colored as rapidly as if the animal had been normal, becoming a deep plum color within three minutes, but very little of the dye had entered the patches at the end of this time. After a further 4 minutes, though, they were no longer to be discerned as such, having merged in the general hue.

The introduction of a second dye into this animal brought out the fact that there was still some persisting local ischemia $1\frac{1}{3}$ hours after the blood had been returned to the body. During much of this period the general circulatory state had been poor. But the rapid distribution of the second dye everywhere in the superficial tissue except in the regions of ischemia clearly proved that these latter were not due to a faulty general condition.

In a second rabbit the phenol red was injected before the bleedings had all been wholly accomplished, with result that depletion was less drastic. The only patching obtained was one traceable to local pressure differences. Like the ischemia unlocalized by any such factor it persisted after return of the blood.

Experiment 6.—A male, white rabbit of 2175 gm. was employed which had been epilated over the trunk and thighs with barium sulphide some days previously. No inflammation of the skin had ensued. The animal was fasted for 24 hours, and then, under ether, the left common carotid was cannulated and obstructed with the same device as in Exp. 5. Prior to closing the incision the tissues were swabbed with novocaine. Fifty-four minutes after the operation had been completed the animal had recovered from the ether and was in excellent state. Now it was bled

28½ cc. in 75 seconds, and 30 minutes later another 21½ cc. in 110 seconds. Thereafter the skin was pallid but still warm, and not blotched. After the lapse of a further 32 minutes 12 cc. of blood was taken in 70 seconds. As each lot was obtained it was defibrinated. Twelve minutes later 8.5 cc. of 4 per cent phenol red was injected into an ear vein in 60 seconds. This caused no symptoms, and the animal, which sat quiet, colored slowly and evenly. So well had the depletion and staining been withstood that eighteen minutes later the rabbit was sniffing about the laboratory floor as if nothing had happened. The general color was now *old rose* save for large patches over the ridge of the backbone and the projection of the knees where the hue was yellower. Since no patching developed in the next twenty minutes a further bleeding of 15½ cc. in 3½ minutes was carried through. Thereafter the patching over back, sides and haunches became more pronounced, *light coral red* as compared with *light jasper red* elsewhere. The animal retained the sitting posture but appeared listless. The entire quantity of blood, 77½ cc., was now reinjected in 185 seconds, thirty-three minutes after the final depletion. The general condition immediately became excellent. Yet the patches persisted unchanged during the next ten minutes, though the red about them became deeper, approximating *eugenia red*. After another seven minutes those over the knees had disappeared and the one along the backbone was fading at the edges. Twenty-four minutes after the return of the blood the color was everywhere the same, slightly deeper than *old rose*. Decolorization, which now began, proceeded swiftly and so evenly as not to suggest that there had ever been patching. The rabbit was *alizarin pink* one hour and forty-four minutes after the return of the blood and still faintly pink three hours after. It was then discarded.

In this instance the removal under local anesthesia of about half of the calculated blood volume did not suffice to bring about a general patching, though the circulation was sufficiently cut down over back and knees for some local acidosis to develop there, as the indicator showed. The abnormal state of affairs was rendered more pronounced by a further bleeding which the animal stood well. And now when all the blood was reinjected and the general condition became excellent the local acidotic patching failed to disappear for a considerable time. That its obduracy was not due to factors effective under normal conditions was shown by the course of the eventual decolorization which took place at the same rate in the regions previously patched as it did elsewhere.

It has been our frequent observation that local pressure conditions of no moment ordinarily will lead in the depleted animal to a localized ischemia indistinguishable, save in situation and in the extent and shape of the patches, from that occurring elsewhere over the body (11).

Exp. 5 yielded an example of the sort, in the patching over the ridge of the backbone, while in Exp. 6 all of the patching was of such type. The ischemia tends to persist like that appearing elsewhere on the body and not evidently referable to any local pressure differences. We have enlarged the observations upon it because of the physiological problem presented by the persistent bloodlessness in general.

As has already been stated the etherized cats of the present investigation were placed on the back or side in postures naturally assumed and with the limbs free. Some regions were necessarily higher than others and in these regions,—over mid abdomen and chest, with the animal on its back, or over the upper side and flank, when it was on its side,—the spotting tended to last longest after return of the blood. When the cat was in the dorsal posture and the hind legs lay symmetrically half flexed with one knee slightly higher than the other, the ischemia persisted longer over this higher knee even though the difference in level was but two or three centimeters. The important influence of local pressure differences, as thus illustrated, was especially well to be seen when one of the legs had been raised for some time.

Experiment 7.—The cat was that of Exp. 3 (q. v.). It lay on the left side, but slightly toward the dorsum. In order to relieve the abdomen and chest from any pressure of the legs that were uppermost,—those of the right side,—a string had been looped around one toe-nail of each and tied to a support in such wise that the limbs were held suspended in the air in the same posture of partial flexion as their fellows. The suspended right foreleg must be dismissed from consideration since it had not been epilated. When the phenol red was injected, after the bleedings, as already described, no stain whatever entered the tissues of the raised hind leg for some little while, but by the time its fellow on the other side had become diffusely orange a well defined, horizontal color demarcation had developed in it just distal to the groin, approximately 5 cm. above the well stained thigh. Above this level the tissues of the leg were pallid and unstained, below it orange. The zone of transition was about 0.6 cm. broad. The femoral vein of the raised leg was noted to be collapsed, that of its fellow distended. Eleven minutes after the return of the blood, when the general body surface was a brilliant pink with scattered orange spots, there was still not the least staining of the suspended leg. The blood pressure had been far above its initial height since the reinjection of the blood. After a further six minutes the line of demarcation had disappeared and phthalein was extending upwards into the skin of the leg, staining it orange (or *apricot buff* in Ridgway's nomenclature). The animal was by now *old*

rose with scattered *apricot buff* patches. Thirty-eight minutes after return of the blood the highest part of the raised leg, the region around the knee, was still only faintly stained (pale buff). This region was 10 cm. above the midline, as the animal lay, but only 3–4 cm. above the highest part of the side. In this part the color had a slight admixture of yellow as compared with the diffuse *old rose* seen elsewhere. The wide-spread spotting had disappeared. The blood pressure had fallen somewhat (from 152 mm. to 134 mm.) but was still above the initial level (120 mm.). The animal had now been under ether for more than two hours.

Experiment 8.—The cat of Exp. 1 (q. v.) served for the observations. It lay on the back under light anesthesia with the legs symmetrically rotated outwards and half flexed. They never became flaccid and hence did not touch the table at all. The right knee was slightly higher than the left. After the injection of the phenol red the tissue for a wide space about the knee was observed not to stain at all, and it remained unstained when the blood had been returned to the body, although the carotid pressure was raised thereby to a much higher level than it had been originally (160 mm. as compared with 142 mm.) and there occurred a wide-spread staining elsewhere. Ten minutes after the injection the superficial tissue about the knees was still uncolored and one could perceive after oiling the skin that this held true of the underlying muscle as well. Yet the main artery of the limb was beating far more violently than usual, owing to the induced plethora, and the pads were red. After another fifteen minutes traces of dye were entering the ischemic tissue, rendering it buff at some places. Elsewhere in the leg one could perceive through the oiled, unstained skin that the large subcutaneous veins were darkly red, with a pink diffusion of dye immediately next them. The animal was in general ruddy, but spotted irregularly with pale orange buff. The blood pressure had remained high. Both legs had attained the same ruddy hue after 44 minutes in all. There was still much spotting over the chest and abdomen. 40 cc. of warmed Ringer's solution was now injected to increase the plethora, and thereafter the spotting soon disappeared.

In these instances the slight circulatory difficulty produced by raising the leg a few centimeters above its fellow sufficed, not merely to render it ischemic after the depletion but to ensure the persistence of the ischemia later, when the blood bulk had been restored and the blood pressure had been brought so high that the circulatory difficulty just mentioned should have been wholly negligible. In Exp. 8 there was an obvious plethora after return of the blood; the main artery of the ischemic limb beat violently, the paw was well stained, and the venous blood deeply so. Yet the tissue previously ischemic remained for some time obdurately bloodless.

Needless to say the factor of local chilling had to be considered as possibly favoring a persistence of the bloodless state, however caused.

The ischemic tissue became cold. So too, if in a less degree, did the body surface patched with ischemia. But that the development of such patching is not essentially dependent upon cooling has been sufficiently shown by immersing animals in oil at body temperature (12). The cats in which its persistence was studied were often long on the table, and care had to be taken to keep them warm in order to prevent shock.

The question whether the spots recur at the same places after a second bleeding is one of great importance for an understanding of them. Many experiments were done upon this theme, a principal difficulty being the long interval required for complete disappearance of the local ischemia after the blood of a first depletion had been returned to the body. It was necessary to recognize and rule from consideration the patching due to local pressure differences (of the sort illustrated by Exps. 7 and 8); for such patching infallibly recurred unless the position of the animal had been changed. Needless to say this recognition and ruling out could not always be perfectly done. Furthermore there were spurious patchings to be discriminated, as where underlying fatty masses made the skin appear pale, or where the circulation was naturally poor, as about the umbilicus. In regions of local inflammation the staining was always far better than elsewhere; but such regions were always readily discriminated. Abrasions with the razor give rise to a staining that is on the actual surface, not beneath it as in the case of the cutaneous staining proper.

The general plan was to use phenol red to disclose the patchings of a first depletion, which were then marked out here and there with dots of india ink; and after restoration of the blood and a second bleeding, —sometimes indeed after return of the blood for the second time,— brom phenol blue was injected. This dye diffuses swiftly and is more easily seen in the tissues than is phenol red; and hence the pallid patches demonstrated with its aid often appear smaller than when the latter dye has been used—the more especially since this turns orange in acidotic tissues. The animals were usually bled to death from the carotids while the ischemic patching of the second depletion was still evident; and the pelt was at once stripped to determine the relation of local features to the patching.

Experiment 9.—The cat of Exp. 1 (and Exp. 8) was kept warm and under light ether until all the patching of the first depletion had disappeared. As the protocol of Exp. 8 shows, the process seemed to be hastened by the increase in plethora brought about through the rapid intravenous injection of 40 cc. of warmed Ringer's solution 54½ minutes after the last blood injection. However this may be, a second series of bleeding was begun 141 minutes after the start of the first. The outlines of fourteen of the characteristic patches induced by the first depletion had been marked on the skin with small dots of india ink. The body surface was diffusely stained *old rose* at the time when the new bleedings were begun. The carotid pressure was 144 mg. Hg, the initial pressure having been 142. Hemorrhages of 43 cc. in 19 minutes now caused it to fall to 78 mm. Within 15 minutes after the initial hemorrhage (of 33 cc. in 4 minutes) the phthalein in the tissues turned toward orange where some of the marked spots had been; and 3 minutes after the second bleeding (of 10 cc. in 1½ minutes) the spots had become accentuated and numerous others were present in unmarked regions. The general color was between *light jasper red* and *alizarin pink*. The body surface was warm. The pressure rapidly recovered to 90 but fell to 82 when a further 3 cc. of blood was taken, 5½ minutes after the second bleeding; and it had not risen 2 minutes later when 6.6 cc. of brom phenol blue was injected in 50 seconds. This raised the pressure to 94 but it had again fallen to 78 when, after 8 minutes more, the entire quantity of defibrinated blood, less 2 cc., was reinjected in the course of 95 seconds. The pressure rose from 76 to 140 mm. in the course of 4 minutes. The general condition of the animal was excellent.

During the interval after the injection of the brom phenol blue and prior to return of the blood a patchy staining of the superficial tissues occurred, superimposed upon that with phenol red. There were numerous scattered splotches into which the new stain did not enter, and the plum color of the regions between was not diffuse but dappled with small spots of dirty yellow which the brom phenol blue had failed to penetrate. Following return of the blood these ischemic yellow splotches stood out pronouncedly, owing to an increased staining of the tissue between them. A count made 3½ minutes after the injection of the second stain showed that 8 of the 14 areas previously marked with ink dots were present as such splotches, but of these 8 two were near the projection of the ensiform cartilage and might have owed their freedom from the blue phthalein to local pressure differences. Five marked areas had stained somewhat, and one was not to be discriminated from its surroundings, being deeply colored. The spots that recurred were not accurately reproduced in the original form, and were in general somewhat smaller. The body surface elsewhere was abundantly spotted. Yet the recurrences within inked outlines were too definitely related to these latter to have been due to chance.

Twenty minutes after the blue phthalein had been given the animal was killed by cutting the carotids. The blood pressure at the time was 128 mm., and the ischemic spots had persisted. At autopsy no local factors to account for them could be found. Numerous others were visible in the furred skin when this was stripped back.

These findings, representative of several experiments, leave no doubt that some of the ischemic patches recur in the same situations after the second depletion, though this is far from being true of all. The injection of brom phenol blue had not been necessary to demonstrate the fact in the instance just given in detail. For the animal was still stained with phenol red after the blood was withdrawn for the second time; and patchy changes toward orange, the color indicative of local acidosis, evidenced a recurrence of the local ischemia in the previous situations.

The circulatory readjustment following the first return of blood was in some cases completed only after the animal had been under ether for several hours altogether. In these cases a singular patterning with brom phenol blue resulted, one wholly different from any observed after depletion under ordinary circumstances, no matter how severe this had been or to how low a level the blood pressure had been reduced.

Experiment 10.—The cat of Exp. 2 still showed some spotting 55 minutes after the first reinjection of blood, and 126 minutes after the initial bleeding. Only after another 38 minutes had the staining become diffuse, the color being then slightly darker than *old rose*. The blood pressure was 166 as compared with 140–145 at the beginning. Now, 206 minutes after the initial etherization, 61 cc. of blood was taken, at three bleedings in the course of 57 minutes, reducing the blood pressure to 72. Twelve minutes later the surface was mottled with buff spots on an irregular ground of *coral pink* and *old rose*. 6.6 cc. of 4 per cent brom phenol blue was injected in 75 seconds, and 5 minutes later all the blood was returned to the body, in the course of 180 seconds. The animal began to stain promptly, a plum blue reticulum enclosing greenish yellow patches averaging about 1 cm. in diameter. A count made 9 minutes after the dye had been given showed that only 6 out of 13 areas ischemic previously, and marked as such with india ink, were unstained by the blue phthalein, the other 7 being as deeply, if irregularly, colored as the tissue anywhere else. One could not be certain that the sparing of even these 6 was due to anything but chance, since ischemic patches were everywhere very numerous. Later the 6 largely lost shape through an irregular encroachment of the plum color. Twenty-eight minutes after the injection of the blue phthalein, and 262 minutes from the time of the initial bleeding the blood pressure was as high as at first (140 mm.). The superficial staining had now assumed a regular pattern, thick purple meshes separating rounded areas of dirty greenish yellow about 1 cm. in diameter. This fish-net mottling persisted practically unchanged throughout the next 40 minutes during which the carotid pressure declined to 130. In the course of the next hundred minutes the mottling largely disappeared, but

not wholly. The experimentation had lasted more than 7 hours. At its end the blood pressure was only 95, the rectal temperature 98.6

The disappearance of the reticulum took place by an entry of the blue dye here and there into the greenish yellow spots, breaking them up into irregular areas 3–4 mm. across. Thus the body surface became thickly strewn with little ischemic patches. These very gradually acquired stain and disappeared.

This experiment, one of several with similar results, yielded evidence of a change in the character of the blood service to the superficial tissue of animals long on the table. But its principal use in the present connection is to illustrate a difficulty met in determining whether local ischemia tends to recur in the same situations.

DISCUSSION

The original aim of the work here reported was to learn whether the ischemic spotting which develops after depletion affects the same regions when depletion is induced once again. Our evidence for the obduracy of the ischemia is the more convincing because unexpected. In the literature one finds few signs to suggest that such a phenomenon exists. The methods have not been fitted to disclose it. Yet bedside practice from immemorial times has allowed for it as a possibility. The old wife customs of chafing bloodless extremities, and applying heat to them, are still in current use to "restore the circulation," on the assumption that restoration might long be delayed without such help.

Zak (13) has described a blanching of the fingers when they are exercised after the circulation to them has been cut off. He believed this due to an active contraction of the small vessels, but Rehberg and Carrier (14) hold that the blood is merely forced from the fingers mechanically. These authors do not deal with Zak's further observation that the vessels contract in a frog muscle exercised while the circulation is stopped, failing for some time to admit blood again when the impediment is removed. Whatever the explanation of this latter phenomenon the circumstances of its occurrence are essentially different from those of the one we have studied. Not so however with the patchy areas of ischemia of the human skin known by the name of Bier's spots (15). These areas of local contraction of the small vessels develop gradually when the circulation to a congested limb is cut off

by means of a pressure cuff; and they are evident as blanchings in the midst of a venous hyperemia. In our animals the local cessation of blood flow was induced by depletion, not by a cuff, and the regions of ischemia were demonstrated with a diffusible stain. The numerous reasons for identifying the ischemic patches of our animals with Bier's spots will be detailed in a subsequent paper. Lewis (16) states that the best-developed spots tend to resist the sudden influx of arterial blood after release of the cuff, which floods the arm with the bright pink of active hyperemia. In our own experience with many subjects and numerous trials this resistance has never lasted long. Most of the blanched spots disappeared at once in the flush, and those which did not faded rapidly from the periphery in the course of a minute or two. But it has seemed unwise to cut off the circulation from the arm for more than 45 minutes. The short period of occlusion when taken in connection with the relatively slow metabolic activities in man as compared with the cat, may explain the differing persistences of the ischemia.

Bier (17) pointed out that the pallor of the dead body is due to a generalized contraction of the small vessels, similar to that manifested locally on the living subject in the blanched spots now called after him; and Lewis holds that the dead whiteness of the fingers in Reynaud's disease is referable to the same cause. Our observations bear out these assumptions, and give ground for a further one, namely that much of the difficulty in restoring the circulation to parts temporarily deprived of blood, as in Reynaud's disease, chilblains and skin threatened with bed-sores, comes from a vascular perversion secondary to the initial ischemia. Whether this perversion occurs in those instances of shock and hemorrhage which continue to show a pallid, cold skin despite transfusion and other active measures remains to be seen. The cause for the phenomenon here reported will be discussed in a later publication. It is scarcely necessary to remark that the length of time during which the ischemia lasted in our experiments did not nearly approach that required to cause frank injury of the tissues. Furthermore the deprivation was the result, not of arbitrary interference with the circulation, but of compensatory readjustments within the organism such as would tend to prolong life.

SUMMARY

In skin regions which have been bloodless for some time, as result of the functional readjustments following upon a reduction of the blood bulk, the ischemia persists long after the blood volume has been restored and the systemic blood pressure has mounted to the initial level or a higher one.

The significance of the finding is briefly discussed.

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THE FINAL RESPONSE OF THE SMALL CUTANEOUS VESSELS

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Plates 24 to 28

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In several previous reports a patchy cutaneous ischemia has been described which develops in animals some time after the blood volume has been markedly reduced (1). Our general procedure to demonstrate it in animals has been to inject a highly diffusible vital dye intravenously after reducing the blood volume by hemorrhage or by anhydremia. Under such circumstances the skin becomes spotted with pallid areas into which the dye has not entered, on an intensely stained ground. The animal in consequence appears piebald. To test for such a state of affairs in human beings one might conceivably compress the main artery to a limb sufficiently to reduce greatly the blood flow to it, and then inject a vital dye into the slight stream still passing the obstruction. Fortunately, there is on record in the literature a phenomenon elicited in man, and still unexplained after thirty years, which nearly parallels both in conditions and consequences our hypothetical experiment. We refer to "Bier's spots." In the present paper the conditions under which these spots arise, their relation to the patchy ischemia of animals, and the significance of both will be discussed.

Bier reported (2) that when the human arm is suffused with blood by obstructing the venous outflow, and all circulation stopped with a tourniquet, a secondary readjustment of the contents of the engorged cutaneous vessels occurs. Pallid spots gradually appear here and there on the violet skin, rapidly increasing in number and size. The blood has in some way been driven out of them. Bier believed that the cutaneous vessels had contracted as result of the stimulus of venous blood, an explanation since voided. The spots have been studied repeatedly in late years but their significance remains uncertain. Rehberg and Carrier (3) discriminated two kinds of patching on the congested arm, the one

pink or light red, and due, so they thought, to the retention of tonus by vessels receiving a little arterial blood through bone-marrow collaterals. Wolf (4) has confirmed the explanation experimentally. The other patches, of blanching, Rehberg and Carrier attributed to capillary contraction as the arm cooled. Wolf has since demonstrated that increasing the local circulation beforehand by heat or friction tends to prevent the spotting; whereas decreasing it, by cold, causes more of the spots to appear. She pointed out that the known factors conditioning the spotting, (a) state of the local circulation prior to the occlusion and (b) the temperature of the skin during it, cannot be the sole influences determining the spots since the utilization of them does not enable one to blanch large areas of skin at will. Recently Lewis (5) has decisively excluded cold as the primary cause for the blanching. He holds it due to vaso-constrictor substances developing in the tissues.

Likenesses of the Spots and the Patching

As the foregoing summary discloses, the conditions which lead to Bier's spots differ in certain respects from those which result in the ischemic patching in animals. In the one case the circulation is completely stopped, whereas in the other it is reduced greatly through compensatory vascular readjustments. In the one the small cutaneous vessels are primarily engorged, and contraction of them becomes evident as it empties them of blood. In the other these vessels are largely empty to begin with and the contracted state is disclosed by the patchy distribution of a vital stain. We have made repeated attempts to elicit Bier's spots in white rabbits, cats and a white pig, but with dubious success. On venous occlusion the skin does not become engorged as in man, and the spotting seen after arterial closure is ill-defined. Hence we have resorted to the intensive study of Bier's spots in human beings, hoping to learn whether the conditions which differentiate them from the ischemic patching are essential or accidental.

Bier's spots are readily produced in most individuals, but best in persons with a fair skin not greatly freckled or tanned. In some the spotting develops pronouncedly, whereas in others it is slow to appear and the blanching is incomplete. Our subjects were volunteers.

In appearance the spots resemble the ischemic patches in stained animals. Both are scattered, of highly various size, small at first and growing by peripheral extension and coalescence into large areas with more or less sharp-cut, serpiginous margins. In animals stained

beforehand with phenol red the development of ischemic patching can be followed by means of the color change attendant upon the resulting tissue acidosis. The bloodlessness of the regions affected was proven by their failure to stain with a second highly diffusible dye injected intravenously. Vital staining was done only after the depletion. Using these methods the progress of the ischemia was studied in a series of animals. It first appeared some minutes after the local circulation had been cut down by hemorrhage and the longer the interval the more widespread and pronounced it was. Many of the spots, though lying in a tissue still fairly served with blood as shown by the staining, were completely ischemic, none of the dye penetrating into them during long periods. The facts are similar for Bier's spots. An interval elapses before they appear, and they gradually become numerous, extensive and more completely blanched.

The Influence of Vascular Distension

Passing now to points of difference, one encounters first the fact that Bier's spots develop in a skin suffused with venous blood, whereas the ischemic patching appears in one already notably anemic. Our first task has been to determine whether the suffusion is a necessary preliminary to the spotting or merely renders it visible. A lead had been provided through the observation that in animals the ischemic patching, though scattered broadcast over the body, tends to be pronounced wherever local pressure differences would act to empty the vessels (6). For example, it is especially marked over the backbone and knees of rabbits crouching naturally, and over the uppermost portions of the prone body. The patching at such situations is distinguishable from that elsewhere only in its shape, as determined by local factors, and in the evident influence of slight differences in tension, or pressure, to determine the localization. We have asked ourselves whether such differences can act to determine the situation of Bier's spotting. That this is the case can readily be shown.

Test 1.—The pressure cuff of a Tycos sphygmomanometer was placed high on the upper arm of a young man (R.), and his blood pressure was taken (102 mm. systolic, 80 mm. diastolic). The subject was seated beside a table with the arm in a natural position over a large mirror which it was kept from touching by a support beneath the cuff and another at the lower edge of the palm. The lower arm

was horizontal with its radial side highest. The pressure in the cuff was brought to 70 mm. for 5 minutes and then abruptly raised to 180 mm. Within 10½ minutes from the beginning of the experiment Bier's spots had begun to appear upon the greatly congested skin of the arm and hand, and after 18½ minutes they were many, large, and greatly blanched. Nearly all were on the upper surface though some few extended as far down as the palmaris longus; and in the mirror a single ill-defined one was visible on the under side of the arm. The thenar eminence showed numerous pallid splotches. The pressure in the cuff was now released.

After an interval of a half hour to allow for the return of normal conditions the experiment was repeated with the subject in precisely the same position save that the lower arm was rotated inwards so that the thenar eminence and radial prominence were on the under side. After 13½ minutes of complete occlusion of the vessels the spotting was as pronounced as before, and again it affected only that part of the arm and hand which was uppermost, precisely the part that had previously been free from it. The thenar eminence, which had become so brilliantly blotched after the first occlusion, showed no spotting whatever nor did that portion of the arm which had formerly been uppermost and was now on the under side (Fig. 1).

Another half hour was permitted to elapse, and then the test was repeated in the position first assumed. The findings were identical with the initial ones (Fig. 2).

In some subsidiary observations made two days later the subject of these tests lay prone on his right side with the left arm stretched horizontally across the mirror, and supported above it as before. The limb was so rotated at the shoulder that the ulnar side of the forearm was highest throughout its extent as had not been the case previously. The many Bier's spots developing in 14 minutes of vascular occlusion were all in the skin of this upper, ulnar side.

In experiments like this one can perceive, in subjects with a thin, fair skin, that during the preliminary venous obstruction the blood tends to accumulate on the dependent side of the arm, only gradually mounting into the skin that is highest. Even when the preliminary period has lasted seven minutes, the upper side of the limb may still be paler and pinker than the lower. After arterial occlusion the difference in hue is soon lost, both sides becoming violet blue; but the difference in shade continues and is accentuated, not merely by the appearance of Bier's spots on the upper side, but by a gravitation of blood from it into the dependent regions, which become more intensely suffused. When the cuff is relaxed bright blood leaps at once into the empty vessels of the skin of the upper side, a brilliant pink hyperemia appearing there. Further down the change lags, and

often one can see, cloudlike masses of dark blood gradually moved, broken up and dispersed in the sweep of bright blood. The entering stream has to displace little or nothing from the cutaneous vessels of the upper side of the arm but much stagnant blood from the lower.

It has been our general habit since Test 1 to study Bier's spots on the propped arm. The position hastens and localizes the spotting. Regularly Bier's spots appear earliest, and are most abundant and whitest, in the skin of the upper side. (Figs. 2, 10, 14, and 15.) Nearly always they are absent from the under side, even when the arterial occlusion has lasted forty five minutes; and usually none have developed more than half way down.

These facts escaped the attention of previous investigators for reasons that become evident when one reads their protocols. The arm with occluded vessels has either been let hang vertically, the local differences in spotting being attributed to anatomical differences in the skin of forearm, palm and fingers,—or it has been rested directly upon a table with result that blood could not accumulate in the skin of the under side, and that this skin could not be seen. The position of the propped forearm which would seem to be most favorable to Bier's spotting is that of horizontal half extension, thumb up. The boneless lower side of the arm into which the blood drains is flaccid under such circumstances, and the spots appear first along the ridge over the radius, in a region that is to say that is emptied not by gravity alone but by some actual pull on the skin over the bone by the heavy, sagging parts.

Although the data thus far presented give good reason for the supposition that suffusion of the vessels acts to hinder rather than to induce Bier's spotting this cannot be concluded forthwith. For the upper side of the arm, gradually emptying of blood, would tend to grow cool sooner than the lower, and cooling favors the spotting.* In the test which follows, the under side of the arm was chilled in water at the temperature optimal for spotting (7).

Test 2.—The same subject (R.) was employed as in Test 1. Preliminary observations were carried out to determine whether the portion of the skin destined to be chilled later would develop spots if the arm were so rotated as to bring it

* We were baulked at first in attempts to elicit blanching under the Cooper Hewitt light for purposes of photography, by the heat that these lights threw out; but when the room temperature was reduced to 70° by a draft of air the difficulty was no longer encountered.

uppermost. For the purpose R. lay on his left side upon a table, with his right arm fully extended and horizontal at the shoulder level. The forearm was propped as usual above a mirror. The olecranon and ulnar border of the hand were the highest points. The pressure in the cuff on the upper arm was raised to 70° for 4 minutes and then kept at 170 mm. for a further 27 minutes. During this period numerous Bier's spots developed on the upper surface of the arm and hand, a few on the sides, but none below.

An hour after release of the pressure cuff the subject was placed at a table, the right forearm horizontal and propped as before, but this time with thumb and radial side up. After 4 minutes of venous engorgement the artery was occluded with 170 mm. pressure, and the arm was lifted by an assistant and placed in a large fish-kettle nearly full of water at 6½°C. Only the lower half of the little finger and the skin of the under side of the hypothenar eminence and forearm were submerged. A single prop sufficed to hold the limb in place, one under the wrist. On the bottom of the kettle was a flat dish full of clean mercury which served as a mirror. The temperature of the room was 20°C., as in the preliminary observations. After 11 minutes of arterial occlusion numerous Bier's spots had developed on the upper side of the forearm, and several on the dorsum of the hand 1½ minutes later. There were some also on the sides of the limb, extending in diminishing number and intensity almost as far down as the water line. After 28 minutes there was still not a single one to be seen in the submerged cutaneous tissue, though now the whole upper side of the forefinger was blanched and there were many large spots on the upper side of the hand and arm. Those situated on the sides had increased but little in size and not in number. The temperature of the water had risen to 8½°C. The arm was now suddenly lifted out and the portion previously submerged was inspected directly for spots. There were none. The cuff was released.

The results of this test, and of one like it on another subject, rule out cooling as the effective cause for the localization of the spots to the upper side of the propped arm. Tests were next undertaken to learn whether the spotting would be more abundant if the cutaneous vessels were emptied artificially instead of by gravity.

Test 3.—The subject K., a healthy young man, systolic blood pressure 110 mm. Hg., diastolic 80 mm., was seated at a table with the arm propped above a mirror, thumb up. The arm was long, slim, thin skinned, fair, with but scanty hair, prominent veins, and almost no skin markings. The pressure in the cuff placed over the biceps region was raised to 70 mm. and three minutes later to 170 mm. at which it was maintained. By this time the numerous large veins had become greatly distended and the skin was everywhere violet. Now as rapidly as possible an Esmarch bandage was wound tightly about the thumb and each finger, beginning with the tip, and it was continued over the hand and up the arm to a point

nearly half way to the elbow. The bandaging was done rather slowly, both because it proved painful owing to the great engorgement, and to allow time for the blood forced out of the tissue to be accommodated in the upper arm. Immediately above the Esmarch a tourniquet was laid on to prevent return of the blood to its original position. This tourniquet consisted of strong rubber tubing which flattened out into a strap 2 cm. broad when wound about the arm. The bandage was then removed. The entire process had taken four minutes. It could now be seen that all the region below the tourniquet was pallid save here and there where the pressure exerted by the bandage had been uneven and a little blood had been caught between its folds, remaining behind as a pale violet blotch. The hand looked like a dead hand. On the portion of the arm above the tourniquet the skin was dusky violet, and a few fresh punctate ecchymoses could be discerned. Seven minutes later it was evident that a considerable quantity of dark blood had passed the barrier of the tourniquet from above, owing to the poor situation of the latter on the thin, bony arm. This returned blood was not distributed evenly, nor was its situation haphazard as had been the case with the little left after the bandaging. It lay in well defined patches suffusing the skin between very numerous spots, characteristic Bier's spots. One of the largest blanched areas covered a part of the under surface of the wrist. There was at this time no spotting whatever on the congested arm above the tourniquet, but a few more ecchymoses had appeared there. Three minutes later the condition was essentially unchanged (Fig. 3). Within another three, however, Bier's spots had developed in the greatly congested skin above the tourniquet. They were small and incompletely blanched, the largest only 6–7 mm. in diameter. Two were on the lower side of the arm. The violet congestion had no greater intensity on this side than on the upper one, for the obvious reason that the vessels were everywhere filled to a maximum. At the time the tourniquet was taken off, after 9 minutes in place, the contrast between the engorged and emptied segments of the arm was extraordinary (Fig. 4). Below the tourniquet there was a leopard mottling, numerous brilliant, white blotches, many of them sharp edged, being scattered everywhere save on the under surface of the wrist. Here and on the dependent thenar eminence some less well defined ones could be made out. Many of the others that were most blanched lay between, not upon, the bony projections of the hand, though in general they were localized in the highest skin regions. The cutaneous tissue between them appeared suffused with blood. The engorgement above the tourniquet was no greater on the dependent side of the arm than on the upper one,—the violet color being the same in both situations; and the few small spots of relative pallor had not progressed in either size or intensity. They cannot be made out in the photograph. No pink spots were visible anywhere at any time. Just before the tourniquet was removed the arm was changed from the horizontal position by lowering the prop beneath the hypothenar eminence 4.5 cm. This was done that gravity might aid in the distribution of the venous blood later. Immediately after the loosening of the tourniquet there was a rush of dark blood into the lower

arm and hand, with an instantaneous partial obliteration of many of the spots previously present. Yet the most pronounced of them remained largely uninvaded by blood during the succeeding three minutes before the pressure cuff was relaxed (Fig. 5). During this period no new Bier's spots developed in the skin of forearm and upper arm, which had been suddenly relieved of its special engorgement; and the few small spots already present did not enlarge or become more distinct.* With the artery freed all previous differences were submerged in the sweep of an active hyperemia. When this had subsided it could be seen that the skin everywhere in the segment that had lain between cuff and tourniquet was evenly speckled with ecchymoses. Below the situation of the tourniquet none were found.

In this test the blood was forced from part of a congested arm into another part which in consequence became so engorged that some of its small vessels ruptured. But even in the extremely engorged tissue Bier's spots appeared. They were as nothing, though, compared with the profusion of great spots over the emptied forearm and hand. The partial obliteration of many of these spots when the venous blood was allowed to return to its original situation proved due, as later tests showed, to the unusually slight tendency of the subject to Bier's spots. Almost none appeared on his arm and hand when they were subjected to ordinary congestion and occlusion. In another individual, with skin reacting more characteristically, the spotting resisted the reflux of venous blood and was accentuated thereafter.

Test 4.—This was carried out like Test 3 but on a middle-aged man (P.R.), whose arm was shorter and thicker, thin skinned but with many freckles. His systolic pressure was 120 mm. Hg, diastolic 80 mm. The artery was occluded after only 2¼ minutes of venous congestion. Four minutes was required to force the blood out of hand and lower arm with the Esmarch bandage, and apply the tourniquet. There still remained an irregular pale violet tint here and there on the wrist and the back of the hand, one so slight that it is not evident in the photograph (Fig. 6). The hand now looked like that of a cadaver. During the succeeding 16 minutes with the tourniquet in place some small, pale patches developed in the skin above it, turgid though this was with the excess blood forced in. The patches were not localized to the upper side of the arm. Below the tourniquet the surface veins gradually filled, though under very slight pressure, and sufficient blood reentered the skin to demonstrate the existence in it of extensive regions of vascular contraction, regions demarcated as such by the failure of the blood to pass into them. These regions were larger and more numerous on the upper side, but there

* For the purposes of photographing Fig. 5 the arm was raised temporarily to the horizontal.

were some in the dependent regions and others that extended into them from above. Especially notable was a blanched area several centimeters in diameter about the ulnar prominence. The entire radial side (upper side) of the forearm was covered by a huge, bloodless spot extending around irregularly to the lower surface of the wrist. Over the region about the knuckle of the index finger was another, less extensive blanching.

Now the tourniquet was released. Just previously the hand had been lowered 2.2 cm. in order to facilitate the return of blood to it, and a little afterwards it was lowered 2.2 cm. more. The height of the prop under the upper arm was not changed. The blood rapidly found its way back, even to the tips of the fingers, and its presence threw into relief on a violet ground the extensive pallid areas (Fig. 7). In the six minutes that elapsed before the circulation was again allowed to enter the limb, these areas became sharper-outlined and progressively more blanched, to a dead white. The one which lay over the radius now extended rapidly into the skin where the tourniquet had pressed, through tissue that still bore the deep marks of it, and into that portion of the arm which had been forcibly congested (Fig. 8). The few small spots previously present in this latter became more clearly marked, but were, to the end, far smaller than those developing in the same region of the same subject under the ordinary conditions whereby Bier's spots are elicited.

When the cuff was relaxed all of the blanched patches were lost at once in the active hyperemia flooding the skin, except that about the base of the first finger, which very gradually faded. In the region above the tourniquet, which had been so greatly congested, the skin was evenly stippled with minute ecchymoses. These were no more numerous where the little blanchings had been than they were elsewhere.

At no time after the Esmarch bandaging were any arterial spots, "red spots," visible, though some had been seen about the wrist during the preliminary period of venous engorgement.

These findings confirm Test 3 and, like it, afford conclusive evidence that the emptier the vessels are, the greater is the tendency to that secondary contraction of them which produces Bier's spots. The other arm of the subject P. R. was submitted to the same test, though with a preliminary period of engorgement lasting 3 minutes; and essentially the same phenomena were observed. Once again an enormous blanched patch developed over the radius, extending from tourniquet to wrist, and rapidly becoming dead white and more sharply demarcated after the reflux of venous blood. Under the ordinary circumstances which lead to Bier's spotting, and after the same time at the same temperature, the subject showed no spots more than $2\frac{1}{2}$ cm. in diameter.

Test 5.—In some subsidiary tests on P.R. the arm was largely emptied of blood before pressure was thrown into the cuff, by raising it above the head and contracting its muscles. Then a pressure of 180 mm. of Hg was abruptly thrown into the cuff and the limb was lowered and propped in the usual horizontal position above a mirror. It was pallid, with but slight distension of the surface veins. After some minutes the dependent side of it became pale violet because of an accumulating small quantity of blood, and here as time went on characteristic Bier's spots were to be seen. All over the upper, radial, side of the arm, which was pale as before, one could make out dimly a great saddle-shaped blanching with ragged edges demarcated by the hyperemia below. After the lapse of nearly half an hour the pressure in the cuff was lessened, till at each inspiration a little arterial blood passed its barrier. This first appeared as a pink flush on the under side of the arm, which, gradually mounting, obliterated some spots and placed others in sharp relief. With due care taken that the influx of blood should be gradual, the last mentioned spots persisted although they were surrounded and demarcated by bright arterial blood. After the arm had become fairly engorged the circulation was once again cut off. Within two minutes many Bier's spots that were entirely new sprang into view, sharp edged and strikingly pale, replacing here and there a pink hyperemia which had only just begun to take on a violet cast.

These various facts prove that suffusion of the vessels is not necessary for Bier's spotting but merely renders it evident to the eye. They show that one of the main differences between the conditions associated with the development of Bier's spots and the ischemic patching in animals, namely fullness and emptiness, respectively, of the vessels, is superficial in nature. Instead of favoring Bier's spotting distension of the vessels acts to hinder it. The spots develop much more pronouncedly when blood channels are almost completely emptied, that is to say when they are in the state that one finds associated with the ischemic patching of animals.

The Influence of the Character of the Blood

The patching develops while there is still a dribble of blood through the tissues, whereas Bier's spots, as ordinarily elicited, appear in tissue from which the circulation has been wholly cut off. Will they develop, like the patching, in skin still supplied with a little arterial blood? It is easy to show that this is the case.

Mention has been made of the "red spots" fed by arterial collaterals which appear about the hand and wrist after occlusion of the

vessels in the upper arm.* Bier's spots can be observed to develop secondarily in the midst of not a few of these. The fact is demonstrable either by following the late changes in "red spots" outlined with dots of india ink, or by inducing relaxation of the vessels of Bier's spots, noting the color of the blood that flows into them and the happenings thereafter. Lewis found that stroking the spots causes them to disappear (8). We have employed either this or gentle tapping to induce relaxation. The color of the blood that surges in discloses whether it is arterial or venous. And there is another, subsidiary criterion. Rehberg and Carrier noted that the blood entering "red spots" blanched by pressure direct wells up from underneath, whereas after pressure elsewhere on the congested arm it rushes in from the violet tissue round about. When the vessels of Bier's spots are abruptly relaxed the direction from which the blood comes is indicative of its derivation.

* The "red spots" appear pale by contrast with the tissue about them. Rehberg and Carrier suppose the tone of their vessels to be maintained by the arterial blood, the result being that they do not become engorged like those about them. Our findings support this explanation, as do those of Lewis on the pallor existing about telangiectases. In five healthy individuals out of eight serving for the study of Bier's spots we have observed local blanchings of special character, well marked, pale areas surrounding little vascular naevi on the congested arm. They came to attention late in a long preliminary period of venous engorgement, and persisted for the first ten or fifteen minutes of arterial occlusion, sometimes longer. So small was the central naevus often that it not infrequently escaped notice before the arm was engorged and then appeared as only a minute point. Even in such instances the surrounding zone of pallor had often a radius of more than one half centimeter.

The blanchings about naevi, instead of intensifying like Bier's spots, tend to disappear as the period of stasis lengthens. That they are attributable,—like the pallor of the "red spots,"—merely to a local persistence of vascular tone in areas unusually well supplied with blood because of proximity to the naevi, was indicated by an involuntary experiment. One of us had a slightly raised, discoid, bright red birthmark, about 1½ millimeters across, in the skin of the flexor surface of the forearm. Pronounced blanching could be elicited for approximately one centimeter around it. As result of the frequent demonstration of the phenomenon clotting occurred throughout the naeval vesssels. The mark became purple and was no longer to be obliterated by pressure. From the time of these changes the blanching could not be induced, nor can it now when the clot has disappeared without return of the abnormally abundant circulation.

Two protocols out of many will suffice.

Test 6.—Subject P. R. The arm was rested on props as usual, in a position midway between pronation and supination, the bases of the thumb and forefinger being highest, and at the same level. The period of preliminary venous engorgement was 7½ minutes. Here and there amid the increasing violet hue of the arm could be seen pink spots, regions where the arterial supply must have been especially direct. These were outlined with fine dots of india ink. Soon after the artery was occluded most of them turned violet; but as the general color deepened one saw amidst it, after 4½ minutes, several persisting pink spots about the base of the thumb and forefinger. After 7 minutes white blotches were developing on the upper side of the arm and hand, and after 14 minutes they were well marked. Special note was made of their relation to the pink spots. Three of the latter about the thumb and forefinger endured for some time as such, but gradually two of them were replaced in large part by complete blanchings without any intervening period of violet coloration. Within the confines of these two, pallor and pinkness were juxtaposed. The third remained pink to the end of the observations. Bier's spots also developed in, or encroached upon, some of the violet regions marked with ink, where the skin had received arterial blood during the initial period of engorgement.

Test 7.—The subject was a young woman, K., with a systolic pressure of 104, diastolic 75. She sat beside a table with the right arm propped above a mirror as usual, thumb up. The arm was plump, tanned on its extensor surface but with thin white skin on the flexor. Few veins or other natural markings were visible. The preliminary period of venous engorgement with the cuff at 60 mm. Hg pressure lasted 6 minutes. As the arm filled one could perceive pink areas here and there amid the pale violet, in special one about the base of the thumb. Now the pressure was raised to 160. A minute later the arm appeared everywhere of the same light violet save on its upper surface where were a few pale flecks,—beginning spots. After 7 minutes of arterial occlusion these spots were much larger and better defined, situated characteristically on the upper side of the limb, especially near prominences on the hand and over the veins of the forearm. There was a very large and pallid one around the base of the thumb. This was now lightly and repeatedly tapped with the smooth, round head of a small hat-pin. Practically at once it melted away and was replaced from underneath by an area of active hyperemia very vivid against the surrounding violet. Three other spots situated respectively at the base of the thumb, about the knuckle of the forefinger, and on the inside of the wrist were similarly treated, and these were also replaced by pink areas. The four largest spots on the forearm were now tapped. They too disappeared, blood rushing in from the sides to engulf them; but this blood was violet not red, and they were completely obliterated for a little while, gradually reasserting themselves. About the knuckle, metacarpal and thumb the spotting also reappeared, developing in the midst of the induced pink patches, then still of

either a frank pink or violet pink color. Tapping them a second time caused two of them to be replaced by the same violet hyperemia as existed in the surrounding tissue. Only the one over the thumb was again replaced by bright blood. The procedure was repeated a third time and with the same results. The cuff was released after 15½ minutes of occlusion. Some of the spots over the thumb and hand remained pallid for many seconds after the rest of the arm had become brightly hyperemic.

The force of the tapping was no greater than proved just sufficient to drive the blood momentarily from normal skin of the same subject.

The method employed in the first of these tests, namely observation without interference, is the preferable one. For, as will be shown further on, stimulation of the cutaneous vessels during stasis often causes an enduring response of them such as cannot but complicate the situation. A survey of many instances enables us to state that Bier's spots develop far more frequently in "red spots" than elsewhere on the congested arm.

The amount of arterial blood which passes the cuff by way of collaterals is exceedingly small (9), and there can be but little circulation through the "red spots" which are the sole cutaneous regions to receive it. Test 7 indicates that when the vessels of these spots close down the arterial blood accumulates behind the obstruction, to flow once again into the tissue when it is removed. Irrespective of this a certainty emerges from the observations, namely, that Bier's spots will develop in regions with tonically contracted vessels receiving a little arterial blood, that is to say in tissue placed under essentially the same conditions which lead to the ischemic patching of animals. True, the vessels of the "red spots" are not nearly so empty as those of the pallid skin which shows this patching. Were they as empty one would expect Bier's spots to occur earlier and be still more pronounced (*vide* Tests 3 and 4).

The major differences in the local states leading to the ischemic patching and Bier's spots have now been dealt with. It is plain that the differences are not basic in nature, but are incidental to the methods of experimentation.

Recurrence and Persistence of the Blanchings

Bier's spots tend to recur in the same situations (10). One reason for this we have disclosed in the influence of local pressure differences.

If the arm be in exactly the same position and state at each test one would expect that the spotting, as determined by such influences, would be in the same places at successive tests. But if the limb be turned ever so little, or the muscular tensions within it altered there would almost certainly be a shifting. This will explain the failure of some observers to note recurrence (11). Only the crudest, most obvious, of the local pressure factors come to the eye of the observer. He cannot perceive those interior strains, stresses and structural differentiations which must largely determine the situation of the blanchings.

Lewis has called attention to the fact that blanchings frequently occur over distended surface veins and may mark out their course. He has tentatively advanced the explanation that the skin is cooled here more than elsewhere by the blood passing up from the hand, though, as he further remarks, thermopyle observations have failed to disclose such a cooling. In the course of our own studies blanching has very often been noted over distended veins and observed to recur there time after time, but this has been only when the veins were upon or near the upper surface of the arm. When the arm was so rotated that they were brought to the dependent side there was ordinarily no spotting whatever in the skin covering them (Figs. 1 and 2), or at most a faint one. It is clear that the spotting over veins merely constitutes a special case of the influence of local pressure differences. But whether these, as determined by postural and structural anomalies, will account entirely for the distribution of the blanchings is doubtful. Bier's spots do not occur over all the distended veins on the upper side of the arm nor even all along the course of a single one. The blanching is not broadcast on the skin of a hand emptied of blood (Tests 3 and 4) but is discrete and sharply localized. Rehberg and Carrier have advanced the view that the small vessels are more sensitive in some places than in others, those of certain areas contracting promptly to stimuli that are ineffective elsewhere.

In numerous experiments reported separately (12) we have sought to determine whether the ischemic patches of animals recur in the same situations. Such of them as were obviously conditioned by local factors regularly did so, and so also with many of which this was not manifestly the case. Others failed to recur, and new ones appeared in different situations. At the time of the work the importance of precisely duplicating the original posture and conditions was not recognized. Both as concerns recurrences in the same situation and in the failures to recur the patches in animals resemble Bier's spots.

During the experiments referred to, which involved the reinjection of blood, it was repeatedly observed that the ischemic patching persisted for a considerable time after the blood volume had been restored and the systemic blood pressure had mounted to or beyond the previous level. Indeed, with the improvement in the circulation the patches often became more brilliantly demarcated than before. One is reminded of Lewis' observation that Bier's spots stand out more strikingly when the arm is placed in warm water, evidently because of a greater engorgement of the vessels about them. He noted furthermore that under ordinary conditions the most pronounced spots disappear only gradually after the pressure cuff is relaxed despite the bright, general hyperemia (13). In our own experience spots have never persisted for more than a minute or two. Their failure to last as do the patchings of animals when the blood bulk has been restored may conceivably be due to the relatively brief period during which the circulation is cut off from the arm and the slow metabolic rate of man as compared with the rat, rabbit, and cat.

The identification of the ischemic patching in animals with Bier's spots in man would seem in view of all the foregoing to be reasonably complete. But there remains the task of determining their cause.

Proximal Causes of the Contraction

What impels the small vessels to contract? There is much to show that when the circulation through them is cut off they become hyperirritable. Török and Rajka (14) have reported that stroking the arm strongly soon after congestion and arterial occlusion leads to an especially great dilatation of the cutaneous vessels. We have noted, both dilatation and prolonged contraction as result of stimuli ordinarily negligible.

Test 8.—The subject was a young woman, M.T., blonde with a plump arm and fair, thin skin, systolic blood pressure 124 mm. Hg, diastolic 80 mm. The arm was propped above a mirror, thumb up. Almost immediately after the pressure in the cuff had been raised to 70 mm. a white spot appeared on the inner side of the wrist, and with the lens a pin-point naevus could be discriminated at its center. Such had been searched for previously but not found. After 7 minutes of gradual venous engorgement the pressure was raised to 160 mm. Already pallid spots had begun to appear on the upper side of the arm. 9 minutes later, when there was a characteristic spotting in this region but none lower than about two-thirds

the way down the side of the arm, stimulation of the skin was begun. It was repeatedly and gently tapped here and there with the rounded head of a hat-pin. When the tapping was within a centimeter of a Bier's spot this as a rule disappeared promptly, though temporarily, by a flushing with venous blood from its surroundings. Tapping on the dependent side of the arm, where there were no blanchings, caused the skin that was directly struck to become a deeper violet than elsewhere. Well defined, greatly engorged areas about 0.5 cm. in diameter could be thus elicited at will. A row of them were in this way brought into existence low along the inner side of the arm (Fig. 9). About each was a broad areola, or halo, approximately 2 cm. wide, of skin paler than its surroundings. This paling developed at the same time that the central spot did. Both persisted for more than fifteen minutes (Fig. 10), being gradually lost thereafter in the general congestion.

Despite a considerable venous pressure, great engorgement, and long stasis the small vessels most unfavorably situated in this subject, those on the lower side of the arm, though engorging more and more with blood from above, still retained some tone as shown by the increased engorgement when they were stimulated mechanically (Figs. 9 and 10). Blood from the surrounding tissue then flowed into them with result that the vessels yielding it were partially emptied of their contents. They did not fill again to the previous extent but remained for a long period more contracted than those elsewhere, as was plain from the local pallor (Fig. 10).

One can suppose that in this instance the mechanical stimulus caused merely a dilatation of the small vessels directly affected, the active contraction of those adjacent occurring in response to an emptying of them into the central patch of skin. Or perhaps contraction was induced by transmitted tension on the skin, a cause suggested for the pallor peripheral to the reflex flare from stroking (15). The first explanation will not cover the following case in which the lightest touch, one insufficient to displace any blood, caused extraordinary blanchings.

Test 9.—The subject R., of Test 1, was seated with the left arm propped horizontally at the shoulder level, above a mirror and rotated internally, with the thumb hanging down and the palm of the hand resting upon a box. The mirror was slanted so that photographs of the skin on the under side could be obtained. The pressure in the cuff was raised to 70 mm. Hg for 4 minutes and then to 170 mm. Five minutes later Bier's spots had begun to appear over the arm and hand. After 25 minutes of arterial occlusion a photograph was taken to show the distribution of spots on the dorsum of the hand between the forefinger and thumb

(Fig. 11). Immediately thereafter the skin was very gently stroked with the rounded head of a hat-pin in a region where the only spotting was a few, very scattered white dots. The stroking was continued for about 15 seconds, over a circular area about 2 cm. in diameter, and it was so light as not visibly to depress the skin. When carried out on the normal hand of the same subject it caused merely a tickling sensation and no evident vascular reaction. A few seconds after it very white Bier's spots sprang into view in the region stroked, so white and so sharply demarcated as to give the appearance that the skin was raised. In Fig. 12 these spots are shown and the center of the area previously stroked is indicated with the hat-pin. After a minute the stroking was repeated in another purple region and with the same results (Fig. 13). There now remained only one large unblanched region on the dorsum of the hand. This in turn was stroked, and it too paled. A photograph 1½ minutes later shows how widespread was the blanching, none of which had faded (Fig. 14). From time to time the dependent thenar region had been stroked, but without eliciting the least change. No blanching appeared there at any time.

After 33 minutes in all the pressure in the cuff was relaxed. The spots promptly disappeared.

The reflection of the under side of the arm and the thenar eminence in Fig. 14 discloses that they were free from spots. But when, shortly after on the same day, the limb was placed with thenar eminence up, and congestion and occlusion were produced, the regions previously free promptly became spotted, whereas the part of the arm that was now lowest showed no blanchings save for one spot associated with a birthmark (Fig. 15).

In this instance intense blanchings sprang into existence, as result of a pressure insufficient to displace the blood from the vessels.

Wolf elicited Bier's spots on arms paralyzed by nerve severance and watched them spread upon sensitive and insensitive skin alike. To Lewis this is proof positive that nervous activity has nothing to do with them. He invokes as their cause vaso-constrictor substances formed within the skin after the circulation has been cut off, and eventually accumulating in such strength that their influence dominates over a vaso-dilator substance also assumed to be present. To account for the prompt disappearance of the blanchings when blood is again let into the arm the further assumption is made that the constrictor substances are so highly diffusible as to be flushed out and away practically at once while the dilator substance, remaining behind, exerts its characteristic effect. The disappearance of Bier's spots on stroking he attributes to a local redistribution of blood, whereby the tissue is relieved of its excessive concentration of vaso-constrictor substances.

Certain of our data make difficult acceptance of these various suppositions. Bier's spots occurring where "red spots" previously were, can be temporarily flushed with arterial blood from the underlying regions by mechanically stimulating the vessels to relax. But they become red for only a minute or so and then blanch once more (Tests 5 and 6). Surely a vasoconstrictor substance with the diffusibility necessitated by Lewis' conception would have been diluted if not carried away by the influx of arterial blood, and blanching could not have recurred so promptly. In the test which follows, the stroking of one Bier's spot led to the appearance of another separated from the first by a belt of engorged skin. The passive transfer of vaso-constrictor substances out of one cutaneous region into another will scarcely explain such a phenomenon.

Test 10.—The subject D. D. (systolic blood pressure 120 mm. Hg, diastolic 80 mm.) had a long, fairly nourished, thin skinned arm, slightly and evenly pigmented, and an unusually pink hand. The limb was propped over a mirror as usual, with the knuckle of the forefinger as its uppermost point. The pressure in the cuff was raised to 70 mm., and to 160 mm. after 7 minutes. 6½ minutes later the whole lower side of the arm was noted to be much more congested than the upper where already numerous scattered pale patches had developed. A minute later a small, circumscribed pale patch about 1 cm. in greatest diameter, lying over the first metacarpal, was repeatedly tapped with the rounded head of a hat-pin. It promptly disappeared, venous blood surging in from the sides, but within less than half a minute a blanched patch suddenly appeared about 1 cm. distant from where the previous one had been and wholly separate from it, over the second metacarpal, that is to say well above the previous spot. This new patch was about 3 cm. in greatest diameter, much more blanched than the original patch, so white and distinct indeed as to suggest urticaria, though none was discernible on palpation. No apparition of the sort occurred elsewhere on the arm at any time, save in response to the stimulus of tapping. This stimulus was applied to several other spots during the period before the cuff was relaxed, after 18 minutes of total occlusion in all. Some of the blanchings disappeared for good, others disappeared and gradually recurred in greatly enlarged form, while in the neighborhood of yet others new and more or less completely separate blanchings sprang into view. Tapping the skin on the upper side of the arm in unblanched regions caused a blanching that proved persistent to appear here and there; but on the under side no response followed. Only on the hand were the Bier's spots numerous or large.

In this and like tests the stimulus which caused relaxation of the vessels of Bier's spots brought about contraction of those in the

neighboring engorged skin. It may well be that throughout the cutaneous tissue a constrictor substance had accumulated which became effectual only when some other stimulus, here a mechanical one, was added to its own. But it will be safest at present to go no further in general explanation of Bier's spots than to suppose that the contractile cells of the small vessels, when no longer properly served by the circulation, become abnormally irritable, responding with contraction (or with dilatation) to stimuli that are ordinarily ineffective.

DISCUSSION

The identification of the ischemic patching in animals with Bier's spots rests upon likenesses which can be summarized as follows:

Ischemic Patches in Animals	*Bier's Spots in Man*
They are blanched areas of skin, similar in appearance and enlarging in the same way. They develop secondarily in tissue no longer well served by the circulation.	
The patches are rendered visible by the vital staining of their surroundings.	The spots are seen because of the blood in the congested skin about them.
The patches appear in skin still receiving a little blood but nearly empty of it.	The spotting is most pronounced in skin inadequately supplied with blood and nearly empty of it.
Both tend to recur in situations previously affected. Local factors which act to empty the vessels are largely responsible for this.	
The patches that have lasted long frequently persist for some time after the blood volume has been restored and the systemic blood pressure has reached the previous level or a higher one.	The spots that are most pronounced,—those usually which have lasted longest,—do not at once disappear when the circulation is restored and the surrounding skin has become actively hyperemic.

It will be seen that like causes in the two cases lead to results that are also alike in their major aspects. Whether the patches and spots are due to physiological mechanisms that are identical in detail remains uncertain. Human skin has some unique characters.

There are additional, minor points of resemblance between the two phenomena. Bier's spots are caused by a contraction of capillaries and venules, which can be followed directly under the microscope. The ischemic patching in animals cannot be so studied, but its long persistence in a skin elsewhere intensely stained after restoration of the blood bulk indicates that the small vessels must be closed down, since otherwise a staining should occur by way of the numerous vascu-

lar anastomoses. The contractile tendency is sometimes strong in Bier's spots, as shown by their development in skin engorged to the degree of ecchymosis (Tests 3 and 4). It is strong too in the ischemic patches, which frequently resist the entrance of blood at high pressure (12). Stroking will cause Bier's spots to disappear temporarily. It has the same effect on the ischemic patching, as shown by disappearance of the localized acidosis secondary to it (16).

One can exclude not a few possible causes for the contraction which gives rise to Bier's spots.

Though elicited ordinarily in an engorged skin the spots are not induced by the engorgement, but on the contrary are rendered greatly more widespread and pronounced by emptying the vessels. It follows that their cause cannot lie in changes undergone by the stagnant blood. They occur more often in "red spots," where there is still a little arterial flow, than in the violet tissue round about; and they sometimes resist the entry of arterial blood (especially when it is under very low pressure) and may rapidly appear in skin rendered pink secondarily by slowly admitting it after a period of occlusion (Test 5). From all this it would seem that neither lack of oxygen nor accumulation of carbon dioxide can be their immediate cause. Lewis' hypothesis that they are referable to highly diffusible vaso-constrictor substances developing in the tissue is, as we have shown, not tenable in its present form. The fact that the blanching is relatively infrequent in regions containing blood, and that it may be largely dissipated by a reflux of venous blood under slight pressure (Test 3) had at first seemed to bear out the idea that the fluid might have an effect to neutralize or dilute constrictor principles present in the tissues. But during the course of tests on the point it was observed, as has just been mentioned, that when blood was let slowly into tissues deprived of it for some time new spots frequently formed, and this no matter whether the fluid had come from other parts of the occluded limb or from the reopened artery. One is forced to conclude that its influence to prevent spotting, as on the under side of the arm, is merely mechanical. And this influence is not always effectual. Some definite blanchings occurred even when engorgement of the vessels was pushed beyond the tolerable limit, as shown by ecchymoses; and under such circumstances the spotting was as frequent on the upper as on the lower surface of the arm, the influence of gravity to localize it having been rendered negligible by a distension of the vessels so extreme everywhere that no shifting of their contents was possible.

Rehberg and Carrier have advanced the view that there is a greater tendency to vascular contraction at some places than at others. And certainly there are regions in which it is sufficient to overcome the maximum pressure that the vessels will stand without bursting (Tests 3 and 4). The cause is not clear for the localization of some of the resultant blanchings in the dependent skin which shows none

when the congestion is less. Perhaps the tension due to the enormous engorgement acts as a stimulus. For, as our tests have shown, the vessels liable to contract are hyperirritable, responding readily to alterations in skin tension. Whether their abnormal irritability is caused by materials retained or lacking, is uncertain, but whatever its cause filling the vessles with fresh blood does not at once do away with it. The influence of local nervous influences to condition its manifestations is far from being ruled out.

Wolf has shown that Bier's spotting occurs independently of central nervous influences (17). The fact that the situation of some of the ischemic patches in animals is frequently determined, like the spotting in man, by slight local impediments to the circulation, and the further fact that the patching thus localized may persist long after the original impediment has been rendered negligible by improvement in the general circulatory condition, both speak for the importance of local causes. Nevertheless the influence of the central nervous system must still be reckoned with. A vaso-constriction of central origin in compensation for a reduced blood bulk may cut down the peripheral circulation to such extent as to render the conditions favorable to a blanching developing secondarily. The ischemic patches on the skin of animals plainly comes about in this way. And we have shown that Bier's spots develop in man not only when stasis is complete but when there is still some little arterial flow to the part, as would be the case under the circumstances of a compensatory vaso-constriction of central origin.

One would expect Bier's spots to appear and coalesce into a widespread blanching wherever the circulation has been greatly reduced for some time. And they are actually observed. The march of events in patients with femoral embolism is as convincing on the point as any laboratory tests (18). Babies partially asphyxiated while being born are purple or mottled (*Asphyxia livida*) if the period of suffocation has not been long, but in severer instances with a feeble heart and low blood pressure the skin is waxen white (*Asphyxia pallida*) (19). These waxen babies usually cannot be revived. That adults ever live long enough in circulatory collapse for a generalized blanching to develop seems doubtful; but it appears very shortly after death, and hence the whiteness of the cadaver (20). The great pallor of the extremities in cases of severe shock or hemorrhage that have lasted for some time may well be due in part to a secondary contraction of the cutaneous vessels. Lewis (21) refers the whiteness of Raynaud's disease to this cause.

There can be no doubt that the vascular contraction which finds expression in Bier's spots and in the ischemic patching of animals is a

response highly fortunate for the organism. It acts to conserve both blood and heat, and this without expenditure on the part of the central nervous system. The gradual pace at which the flow is resumed when the ischemia has endured for a long while (22) may not be without its benefits, since thereby the accumulated metabolic products of ischemia are released only little by little into the blood stream.

In a subsequent paper the influence of the final vascular contraction upon certain forms of vaso-dilation will be discussed.

SUMMARY

A study of the blanchings (Bier's spots) which develop in human skin deprived of circulation has proved them referable to the same general causes that lead to ischemic patching of the skin of animals, and that they are conditioned by the same factors. Both are expressions of a secondary contraction of vessels that have become hyperirritable owing to an inadequate circulation. The contraction is favored by emptying the vessels, but it occurs pronouncedly even when there is a trickle through them of arterial blood. In the case of man the contractile impulse is sometimes so great as to overcome the maximum pressure that the vessels will support.

The significance of the observations is discussed.

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EXPLANATION OF PLATES

PLATE 24

FIGS. 1 and 2. To show the localization of Bier's spots to the upper side of the propped arm (see Test 1). The photographs were taken from above and in front of the arm, to include the mirror reflection of its under surface.

In Fig. 2 typical spots can be seen along the course of the radial basilic vein, which did not occur when the limb was so placed that this vessel lay on its under side (Fig. 1). (In Fig. 1 there is dimly visible on the under side of the arm a pale birthmark outlined with dots of India ink.)

PLATE 25

FIGS. 3, 4, and 5. Bier's spotting is favored by emptying the vessels and prevented by forcibly engorging them (see Test 3). The blood from the hand and lower part of the congested arm with circulation occluded had been forced into the upper part and largely retained there by means of a tourniquet.

PLATE 26

FIGS. 6, 7, and 8. Bier's spotting is favored by emptying the vessels and prevented by forcibly engorging them (see Test 4). In this subject the tendency to vascular contraction was far greater than in that of Test 3. Between the wrist and the marks of the tourniquet an immense blanched area can be seen which resisted the return of the venous blood.

PLATE 27

FIGS. 9 and 10. The effect of local mechanical stimulation upon the vessels of the congested arm with occluded circulation (see Test 8). The arrows point to three areas low on the side of the limb, which had been repeatedly tapped with the head of a hat-pin. Where this struck there is a greatly increased congestion, while round about there is pallor. Fig. 9 shows the immediate response, and Fig. 10 that it was present unchanged after 15 minutes.

PLATE 28

FIGS. 11 to 15 inclusive. To show that Bier's spots can be made to appear by light stroking of the skin (see Test 9). In Figs. 12 and 13 the point of the hat-pin indicates the regions previously stroked and now occupied by spots. In Fig. 14 the blanched areas have extended and coalesced in response to strokings between them.

In Figs. 14 and 15 mirror reflections are included, since these demonstrate that the spotting was localized to the upper side of the arm irrespective of the position of the latter.

The photographs of Figs. 11 to 15 were taken by Cooper-Hewitt light.

(Rous and Gilding: Final response of cutaneous vessels)

(Rous and Gilding: Final response of cutaneous vessels)

(Rous and Gilding: Final response of cutaneous vessels)

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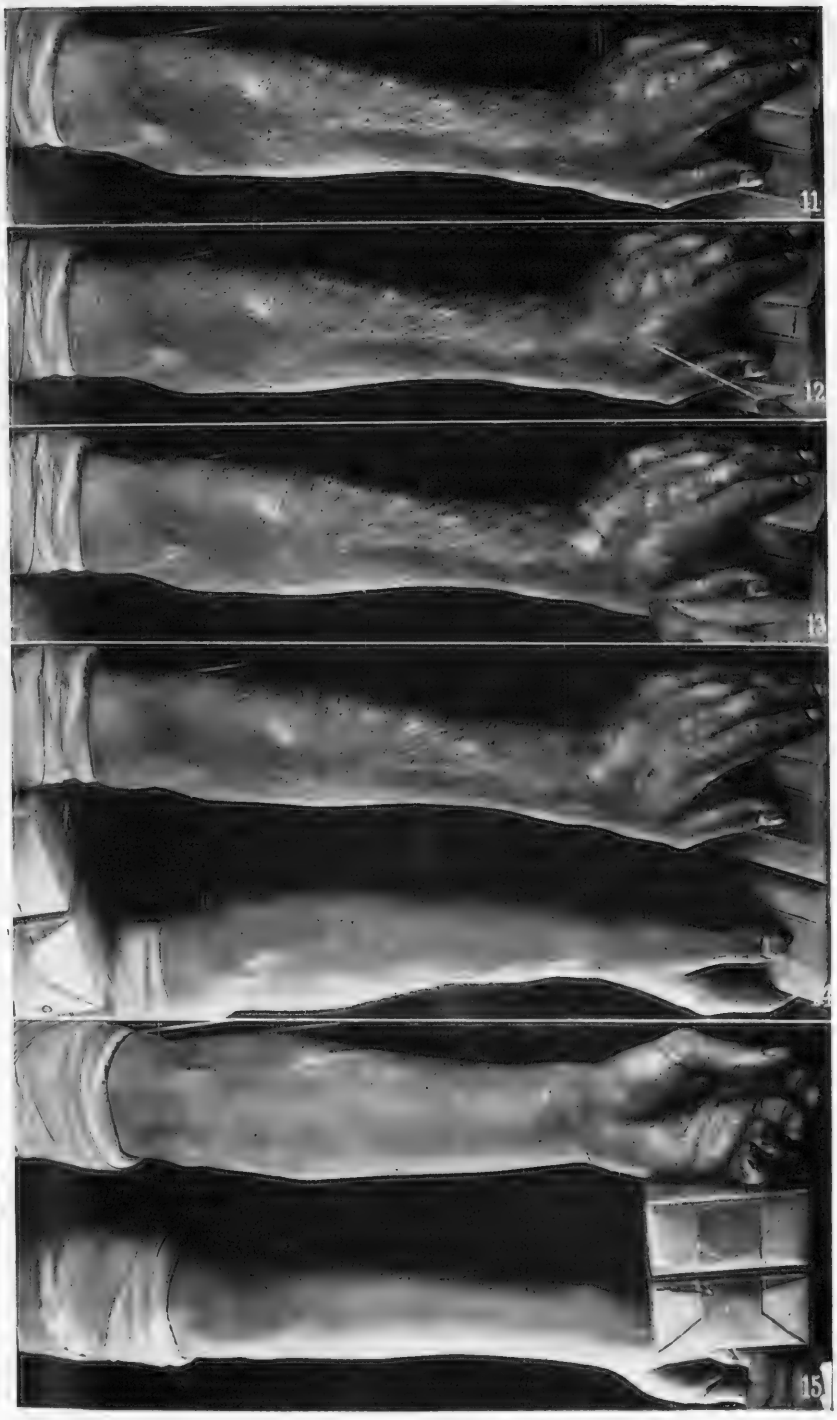

(Rous and Gilding: Final response of cutaneous vessels)

STUDIES ON BACILLUS TYPHOSUS TOXIC SUBSTANCES

III. The Effect of Sera upon the Injury Producing Factors of the Phenomenon of Local Skin Reactivity

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(Received for publication, July 13, 1929)

In previous communications (1–8) there was described a phenomenon of local skin reactivity to culture filtrates of bacillus typhosus and other microorganisms. The reactivity was induced by the injection of a filtrate into the skin of a rabbit. If 24 hours later an intravenous injection of the same filtrate was given to the rabbit, there appeared an extremely severe hemorrhagic necrosis at the site of the previous injection. The factors determining the local skin reactivity were termed "skin preparatory factors" and those responsible for the local injury following the intravenous injection were called "reacting factors." Features characteristic of this phenomenon as well as certain properties of the skin preparatory factors were described. One of these properties was the specific neutralizability by immune sera of the *skin preparatory* factors (2, 3). The object of the work reported in this paper was to determine the effect of sera upon the *Bacillus typhosus reacting* factors.

EXPERIMENTAL

Titration of Bacillus typhosus Reacting Factors against a Constant Amount of Skin Preparatory Factors

Filtrates of washings of 20 to 22 hour old *Bacillus typhosus* cultures on plain agar (T_L "agar washings" filtrates, Batch "A_7") were employed. The method of preparation of the filtrates has already been reported in detail (6).*

* These filtrates were chosen in preference to those previously employed (2) because under the conditions of their preparation, there was present less extraneous material and in all probability less bacterial autolysis.

Preliminary titrations established the fact that a dose as small as 0.25 cc. of the filtrate previously diluted to 1:100 was capable of preparing the skin of some of the rabbits to the phenomenon providing sufficiently large intravenous doses were subsequently used. Because of the high dilution, however, there occurred individual fluctuations (2, 6, 8). In order to obtain a high degree of reactivity necessary for the work to be described, rabbits were prepared with many multiples of the above indicated minimal skin preparatory dose and the reacting factors were titrated in these rabbits as follows:

Protocol I. Fifteen rabbits received each one intradermal injection of 0.25 cc. of the undiluted filtrate "A_7." Twenty-four hours later the rabbits were divided into 3 groups of five. Each group received a single intravenous injection of the same filtrate. The first group received 0.05 cc., the second group 0.01 cc. and the third group 0.005 cc., respectively, per kilo of body weight. Four and one-half hours later, there were observed severe hemorrhagic reactions in 5 rabbits of the first group, in 4 rabbits of the second group and only in 1 rabbit of the third group.

From this titration, it will be seen, that 0.01 cc. of filtrate "A_7" per kilo is the minimal dose of reacting factors capable of eliciting reactions in a large percentage of rabbits prepared with 0.25 cc. of the undiluted filtrate.

Protocol II. This experiment was identical with the experiment of Protocol I, with the exception that each of the 15 rabbits was prepared by one intradermal injection of 0.25 cc. of the filtrate "A_7" previously diluted 1:4 instead of the undiluted. In this experiment there were observed severe hemorrhagic reactions in 3 rabbits of the first group and in 1 rabbit of the second group. The third group showed no reactions.

As is seen from this experiment, 0.05 cc. per kilo is the minimal dose of reacting factors capable of eliciting reactions in a large percentage of rabbits prepared with 0.25 cc. of the filtrate "A_7" previously diluted 1:4.

For the neutralization experiments about to be described, several multiples of the above indicated minimal doses of reacting factors were employed in order to be assured of a high percentage of positive reactions in rabbits. Furthermore, inasmuch as a certain proportion of rabbits was spontaneously resistant to the phenomenon (1, 2) at least five rabbits were employed for each neutralization experiment.

One batch of the filtrate (A_7), stored in the refrigerator, was employed throughout the work. The filtrate was first used approximately two weeks after its preparation and this use was continued for two months afterwards. Since there were observed changes in the potency of different batches of filtrates from time to time, four con-

trol titrations of the reacting factors were carried out during the two months. In addition, whenever a serum neutralization experiment was carried out, a control group of 4 to 6 rabbits was injected with the filtrate alone. These experiments definitely proved that the filtrate "A_7" did not suffer any change in potency during the two months in which this work was carried out.

Neutralization of the Bacillus typhosus Reacting Factors by Specific Sera

The antityphoid sera were obtained from horses and rabbits.

The immunization of horses was carried out by simultaneous weekly subcutaneous and intravenous injections. Filtrates of 6 day old *Bacillus typhosus* cultures in tryptic digest broth, as well as toxic substances precipitated by ammonium sulphate (8) were injected subcutaneously. Unwashed *Bacillus typhosus* heat killed vaccines were injected intravenously. The length of time during which the immunization was continued before each bleeding, is indicated in following tables. The doses were increased by 25% each week. Previous to the bleeding on the eighth month, some of the horses (e.g. #2 and #4) were receiving the following rather large doses: 400 cc. of vaccine, intravenously, 1,200 cc. of filtrate, subcutaneously, a suspension of 9 grams of the precipitated toxic substances (a yield from 1,000 cc. of tryptic digest broth culture filtrate), subcutaneously, and about 400 cc. of the vaccine intravenously.

Rabbit serum R201 was a mixture of sera of 5 rabbits immunized by a method similar to that employed for horses. The injections were carried on for 4 to 6 weeks, 25 cc. of the filtrate was the largest subcutaneous dose and 20 cc. of the vaccine was the largest intravenous dose.

Normal rabbit and heterologous horse sera (Meningococcus and Erysipelas therapeutic sera) were also employed.

The experiments were conducted in a manner similar to that described in previous papers.

One area of the skin of the abdominal wall of a rabbit was injected with 0.25 cc. of the undiluted filtrate "A_7" or 0.25 cc. of the filtrate previously diluted 1:4. Twenty-two to twenty-four hours later a single intravenous injection of the filtrate or of a mixture of the filtrate with a given serum was made. The mixtures prepared on the morning of the experiments, as well as the filtrate alone were incubated in the water bath at 37°C. for one hour. The precipitate in the mixtures was broken up by shaking immediately before the injection. The readings of the reactions were made 4½ hours after the intravenous injection. A rabbit was recorded "positive" when the prepared area of the skin showed severe hemorrhagic necrosis. "Doubtful" rabbits were those showing mild punctiform hemor-

TABLE I

The Effect of Sera upon Bacillus typhosus Reacting Factors

Intradermal dose of "A_7"	Mixtures made in Vitro		Agglutination titer of sera for *B. typhosus*	Total of rabbits tested	No. of positive rabbits	No. of negative rabbits	No. of doubtful rabbits	No. of deaths
	Intravenous dose of "A_7" per kilo	Intravenous dose of serum per kilo						
0.25 cc. dil. 1:4	0.3 cc.	—	—	31	19	0	1	11
0.25 cc. undiluted	" "	—	—	57	41*	1	1	18
0.25 cc. dil. 1:4	" "	Normal rabbit #282 Serum (0.3 cc.)	1:16	19	13	0	3	3
0.25 cc. undiluted	" "	Normal rabbit #169 Serum (0.3 cc.)	Negative	5	3	0	0	2
0.25 cc. dil. 1:4	" "	Therapeutic Horse Meningococcus Serum 148/438 (0.3 cc.) N. Y. Board of H.	1:64	9	6	2	0	1
0.25 cc. undiluted	" "	Lederle anti-Erysipelas horse serum (0.25 cc.)	Negative	15	9	0	1	5
0.25 cc. dil. 1:4	" "	**Horse anti-typhoid serum (0.3 cc.)	1:25.600	7	0	7	0	0
0.25 cc. dil. 1:4	" "	**Horse 62/4 anti-typhoid serum (0.3 cc.)	1:25.600	14	0	14	0	0
0.25 cc. undiluted	" "	**Horse 62/4 anti-typhoid serum (0.3 cc.)	1:25.600	9	0	9	0	0
0.25 cc. undiluted	" "	Horse 62/4 anti-typhoid serum diluted 1:5 (0.3 cc.)	1:25.600	7	3	4	0	0

* 4 rabbits were dead at the time of reading, but the reactions were distinct and well-defined.

** These samples of blood were taken after 7½ months of immunization.

rhagic areas of dark red appearance. In the "negative" rabbits, no reactions were seen. Some of the rabbits died in the course of the 4½ hours following the intravenous injection. Their deaths were recorded. If the dead rabbits showed

no reactions or a diffuse discoloration of the skin, no readings were recorded on the protocols. When, however, there was observed a well-defined reaction, the result was noted.

Table I represents a summary of the results obtained.

As is seen from this table, the skin and intravenous doses employed elicited severe hemorrhagic reactions in about 95% of the surviving rabbits. On the other hand, 30 rabbits which received a mixture of the same amount of the filtrate with certain immune sera (Horses 3 and 4) showed no reactions. The experiment establishes beyond question the fact that *Bacillus typhosus* reacting factors can be neutralized by immune sera.

When a mixture of the filtrate with the immune serum (Horse 4) previously diluted 1:5 was injected, there were obtained reactions in 3 rabbits out of 7. If the high percentage of positive results obtained in the control group is kept in mind, it becomes evident that the 1:5 diluted serum produced neutralization of the reacting factors but did so irregularly. The irregularity of neutralization with this dilution may be explained by assuming that the mixture contained a small amount of free reacting factors or that there was only partial neutralization of these factors so that only these rabbits possessing a high degree of reactivity were able to respond with reactions.

It will furthermore be seen that sera possessing no normal agglutinins, or agglutinins of a low titer for *Bacillus typhosus* (282, 169 and Erysipelas serum) had no neutralizing potency. With one serum (148/438 antimeningococcus horse serum) which had normal agglutinins for *Bacillus typhosus* in dilution 1:64, there was a suggestion of irregular neutralization.

The immune sera had unquestionably a remarkable protective potency against the mortality induced by the filtrate.

Titration of the Neutralizing Potency of Immune Sera

It was important further to determine whether the neutralizing potency of the sera could be quantitatively measured. In the experiments summarized in Table II, a constant amount of serum was titrated against increasing quantities of reacting factors.

As is seen from Table II, a given amount of serum was able to neutralize increasing amounts of the filtrate. The neutralization

TABLE II

Titration of the Neutralizing Potency of Anti-typhoid Sera

Intradermal dose of "A_7"	Mixtures made in Vitro		Agglutination titer of sera for *B. typhosus**	Length of antityphoid immunization	No. of rabbits tested	No. of positive rabbits	No. of negative rabbits	No. of doubtful rabbits	No. of deaths
	Intravenous† dose of "A_7" per kilo	Intravenous dose of serum per kilo							
0.25 cc. undiluted	0.5 cc.	R "201" (0.25 cc.)	1:6400	6 weeks	5	0	4	0	1
" "	0.75 cc.	" "	"	" "	5	0	2	3	0
" "	1 cc.	" "	"	" "	5	2	1	0	2
" "	0.5 cc.	Horse 2 (0.25 cc.)	1:25.600	7½ months	5	0	5	0	0
" "	1 cc.	" "	"	" "	5	0	2	2	1
" "	1.25 cc.	" "	"	" "	5	3	0	1	1
" "	0.5 cc.	Horse 5 (0.25 cc.) Bleeding 56	1:25.600	5 "	5	0	5	0	0
" "	1 cc.	" "	"	" "	5	0	5	0	0
" "	1.5 cc.	" "	"	" "	5	5	0	0	0
" "	1.5 cc.	Horse 5 (0.25 cc.) Bleeding 63	"	7½ "	5	2	3	0	0
" "	0.3 cc.	Horse 4 (0.25 cc.) Bleeding 3	1:1600**	6 weeks	5	3	2	0	0
" "	0.5 cc.	" "	"	" "	5	4	0	0	1
" "	0.75 cc.	" "	"	" "	5	2	0	1	2
" "	1 cc.	" "	"	" "	5	3	0	0	2
" "	0.5 cc.	Horse 4 (0.25 cc.) Bleeding 62	1:25.600	7½ months	15	0	15	0	0
" "	1 cc.	" "	"	" "	10	0	10	0	0
" "	1.25 cc.	" "	"	" "	5	2	3	0	0
" "	1.5 cc.	" "	"	" "	5	1	4	0	0
" "	1.75 cc.	" "	"	" "	5	2	0	1	2

* Agglutination tests were made at the time of the experiments.

** The titer was the same shortly after bleeding.

† Previous experiments have shown that these doses consistently produce reactions in animals prepared with 0.25 cc. of undiluted filtrate "A_7."

was considered "consistent" if obtained in all rabbits tested (a minimum of 5 animals). As the amounts of reacting factors were increased, neutralization was obtained irregularly, i.e., in some of the

rabbits only. The maximum amount of reacting factors "consistently" neutralized by 0.25 cc. of a given serum was tentatively used to compute the neutralizing titer of a given serum. Thus, if a 0.25 cc. of a given serum is able to consistently neutralize 0.5 cc. of "A_7" (R 201, Table II) and irregularly neutralize 0.75 cc., its titer must be between these two figures. Since 0.01 cc. per kilo of reacting factors is the minimum dose (page 514), the serum has been able to neutralize between 50 and 75 minimal reacting doses, the titer, is therefore, approximately 60.

In these titrations 0.25 cc. of serum was used instead of 1.0 cc. in order to keep down the volume to be injected. The titrations in terms of 1.0 cc. must be worked out experimentally.

It will also be seen in Table II (bleedings 3 and 62, horse 4) that the titer of the neutralizing antibodies in the serum increased in the course of immunization.

As indicated in the text, the purpose of the procedure described was twofold: The first object was to establish the fact that sera can be prepared which have the power to neutralize the *Bacillus typhosus* reacting factors. The second object was to outline a method for titrating the potency of the sera. It is obvious, however, that it is necessary to develop a permanent standard for ultimate comparisons since batches of toxic substances vary in their potency. Work is under progress with this object in mind.

CONCLUSIONS AND SUMMARY

It has been demonstrated that many multiples of minimal doses of *Bacillus typhosus* reacting factors can be neutralized by specific immune sera.

The potency of a given serum can be conveniently titrated against increasing amounts of reacting factors.

If the immune serum is diluted or if the amount of the reacting factors is too large for a given amount of serum, there is obtained neutralization but only irregularly.

Normal and heterologous sera (therapeutic meningococcus and erysipelas horse sera) free of normal agglutinins or possessing normal agglutinins of a low titer (1:16) for *Bacillus typhosus* are not able to neutralize the reacting factors. There is obtained questionable

neutralization with a serum possessing normal *Bacillus typhosus* agglutinins in dilution 1:64.

The titer of the neutralizing antibodies increases in the course of immunization.

Immune sera exercise a definite protection against the mortality induced by intravenous injection of *Bacillus typhosus* culture filtrates.

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CHEMO-IMMUNOLOGICAL STUDIES ON CONJUGATED CARBOHYDRATE-PROTEINS

I. The Synthesis of *p*-Aminophenol β-Glucoside, *p*-Aminophenol β-Galactoside, and Their Coupling with Serum Globulin

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The problems of the relationship between chemical constitution, physiological effect and biological specificity, which found their origin in the study of the active principles of certain natural drugs, have become so absorbing and so embracing that they have attracted and held the interest of the chemist, the pharmacologist, and the immunologist alike. The question of protein specificity (1), and that of whether it is possible to change specificity by altering the protein molecule through chemical means, have, in particular, engaged the minds of many investigators. On the other hand, the rôle which carbohydrates play in the phenomena of immunity has only recently been disclosed, despite the fact that the presence of these substances in bacteria and yeast has long been known.

Some years ago, Dochez and Avery (2) observed that filtrates of pneumococcus cultures contained a stable substance which reacted specifically with antipneumococcus serum of the homologous type. This observation led to the isolation and identification of these specifically reacting substances from the three specific types of Pneumococcus (3), and from the three types of Friedländer's bacillus (4). Since then, other investigators have isolated similar substances from various other species of bacteria (5).

These type specific substances fall into the class of carbohydrates. They are unusual carbohydrates in that each contains a sugar acid as an integral part of its complex molecule. Immunologically they belong to that important group of specifically reactive but non-antigenic substances which Landsteiner has termed *haptens*.

One of the striking characteristics of these bacterial carbohydrates is their failure to produce antibodies when injected into the animal organism, though in the state in which they occur in the bacterial cell they are not only type specific, but are also antigenic as well. In order that the bacterial polysaccharide may be effective as antigen it is believed, therefore, that it must be combined with another cellular constituent—possibly a protein—to form a complex and easily dissociable antigen. The nature of the substance which confers antigenicity upon the carbohydrate substance of Pneumococcus and the character of its union, are problems which as yet have not been solved.

The products of hydrolysis of these specific carbohydrates have been studied in detail (6). All except the polysaccharide of Pneumococcus Type I yield glucose on hydrolysis. The carbohydrate of Pneumococcus Type III and of Friedländer bacillus Type A yield on hydrolysis isomeric aldobionic acids (glucose-glucuronic acid) in addition to glucose. Evidence has likewise been secured which indicates that the specific carbohydrates of Type II Pneumococcus and of Type B and C Friedländer bacillus also contain other aldobionic acids.

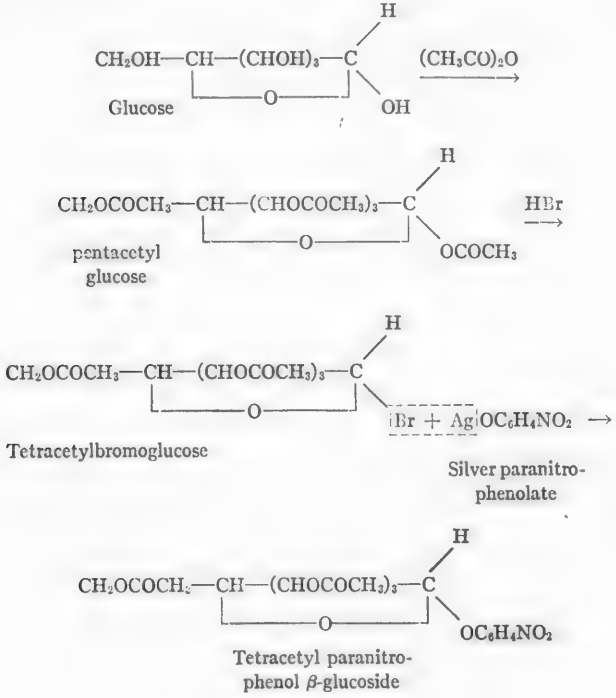
The selective specificity of encapsulated organisms, such as Pneumococcus and Friedländer bacillus, seems to depend primarily on the hapten part of the hypothetical complex antigen. In all of the specific carbohydrates studied thus far, the invariable presence of *isomeric* aldobionic acids seems to indicate that particularly the acid group (carboxyl group) and its stereochemical relationship to other groups of the intact polysaccharide molecule, which in each instance must necessarily be different, has a profound influence in orienting specific response. This question, however, will be dealt with in greater detail in a later communication.

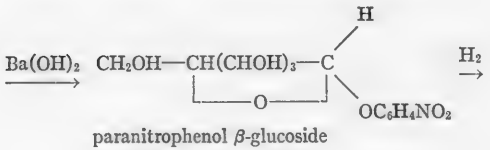
For the purpose of studying the rôle which simple sugars of different spatial configuration might play in altering the specificity of proteins, it was thought that it might be possible to combine these different sugars with a given protein, and to observe specific differences in antigenic properties of the substituted compounds.

The problem thus becomes two-fold, first, to find some means of combining the sugar with the protein, and second, to synthesize the appropriate derivative. Pauly's (7) fundamental method was made use of in combining glucose and galactose to serum globulin.

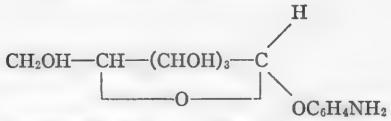
Some years ago Pauly (7) described a color test for the detection of tyrosine and histidine in the hydrolytic products of proteins. This test was dependent upon the formation of colored azo dyes produced by the coupling of diazobenzene sulphonic acid to the aromatic nucleus of amino acids. The coupling of diazobenzene sulphonic acid and other derivatives of phenyldiazonium chloride can be brought about not only with hydrolytic products of proteins, but with the intact protein molecule as well (8). This reaction has been used by Landsteiner (9) in preparing a number of substituted proteins,—"azo proteins,"—and in a series of experiments he studied the immunological reactions of a great variety of differently substituted proteins. By this method he was able to differentiate between the dextro and laevo forms of phenyl-amino-acetic acid. In the same communication (9*f*) he indicated the bearing of his observations on the serological specificity of bacterial carbohydrates.

The second problem, that of preparing a sugar derivative adaptable to this principle, was solved by the following synthesis:



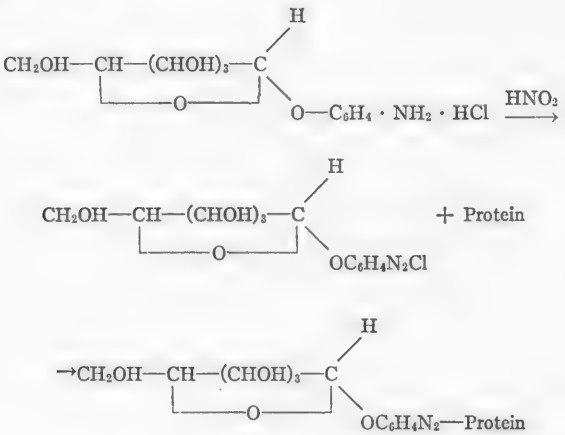


paranitrophenol β-glucoside

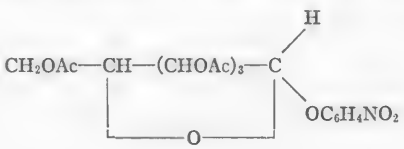


paraaminophenol β-glucoside

The end product of this series of reactions may then be converted into the corresponding diazonium derivative and coupled with protein in the presence of dilute alkali:



The most difficult step in the synthesis, the coupling of p-nitrophenol with the acetobromo-sugar, was accomplished by shaking a solution of the latter in xylene with the silver salt of p-nitrophenol. In this manner, fair yields of the glucoside



were obtained. The preparation of p-nitrophenol glucoside from this derivative, and the catalytic reduction of this compound to its amino derivative proceeded very smoothly and nearly quantitatively. The coupling of the diazonium phenol hexoside to protein takes place readily and rapidly in the presence of N/100 hydroxide.[1]

Thus with the aid of *p*-aminophenol glucoside and the corresponding galactoside two different hexosides have been attached to the same protein, yielding complexes which exhibit different optical properties and which yield reducing sugars on hydrolysis. It should be possible to attach any aldose or ketose to native protein provided the intermediary glucoside can be synthesized. Owing to the occurrence of isomeric aldobionic acids in specific bacterial polysaccharides, which are believed to play an important rôle in orienting the antigenic specificity of encapsulated bacteria, it would seem to be of even greater interest to couple sugar acids to proteins in order to study the antigenic response of such substituted proteins. We are at present attempting to synthesize such derivatives.

EXPERIMENTAL

1. *Pentacetyl glucose* and *Pentacetyl galactose.* These compounds were prepared by acetylation of the corresponding sugar with pyridine and acetic anhydride at 0°C. Both products were recrystallized from alcohol. The pentacetyl galactose melted at 146°C.

2. *Acetobromo glucose* and *Acetobromo galactose.* These derivatives were prepared by the method of Fischer (12).

3. *Silver p-nitrophenolate.* 1.2 mols of recrystallized dry p-nitrophenol were dissolved in absolute ethyl ether. 1 mol of freshly precipitated dry silver oxide was added. The mixture was shaken for 18 hours at room temperature together with a large quantity of glass beads. At the end of this time a brick red compound separated. This was filtered on a hardened paper, washed repeatedly with

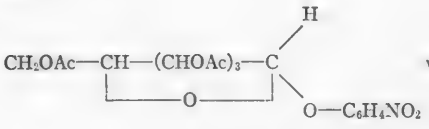
[1] There have recently appeared two papers by Klopstock and Selter (10) wherein they contend that neutral solutions of derivatives of phenyl diazonium chloride do not combine with, but are merely "adsorbed" by protein. This contention seems to have been refuted, however, by the experiments of Heidelberger and Kendall (11). It might be mentioned here again that the presence of alkali is not always necessary for the coupling of phenyl diazonium chloride derivatives to aromatic nuclei. In fact, the formation of some azo dyes is brought about by coupling diazonium chloride derivatives not only in alkaline, but in neutral, and even in acid medium as well.

absolute ether, and dried in a vacuum desiccator over phosphorus pentoxide and paraffin for 4 weeks. The compound is probably a mixture of the true silver salt of p-nitrophenol, $AgOC_6H_4NO_2$, and of its isomeride, $O{:}C_6H_4{:}NOOAg$. Two distinct crystalline forms may be seen under the microscope.

Analysis: 0.2000 gms. substance gave 0.1181 gms. AgCl when analyzed by the method of Carius.

Calculated for $C_6H_4NO_3Ag$ — Ag: 43.85 per cent. Found 44.40 per cent.

4. *Tetracetylnitrophenol β-glucoside:* 50 gms. of aceto-bromo glucose were dissolved in 500 cc. of anhydrous xylene. To the mixture was added 10 gms. of the silver salt of p-nitrophenol. After shaking with dry glass beads for 30 minutes a second portion of 10 gms. of the silver salt was added, and finally after another 30 minutes a third portion was added. The mixture was removed from the shaking machine, filtered on a hardened paper, and the precipitate was washed with small portions of xylene. The filtrate was now concentrated to 50 cc. *in vacuo*. 100 cc. of absolute ethyl alcohol were added, and then the mixture was further concentrated. The addition of alcohol, followed by evaporation, was repeated three times. The final concentrate was taken up in 200–300 cc. of absolute alcohol and the flask was placed in the ice box. After 24 hours the glistening pale yellow crystals of the glucoside were filtered from the deeply colored mother liquors. They were dissolved in about 250 cc. of hot absolute alcohol, and boiled with a little norite. The solution was filtered through a hot water funnel. After 24 hours in the cold the nearly snow-white crystals were filtered from the pale yellow mother liquid. A second crystallization yielded about 9 gms. of pure glucoside,



which melted at 172–173°.

$$[\alpha]_D^{25^\circ} = \frac{-\ 0.86 \times 100}{2 \times 1.055} : -\ 40.8^\circ \text{ (in chloroform)}$$

Analysis: 6.595 mgs. substance: 12.370 mgs. CO_2 and 2.940 mgs. H_2O

$C_{20}H_{23}O_{12}N$. Calculated: C = 51.16 per cent, H = 4.94 per cent.

Found:— C = 51.15 per cent, H = 4.98 per cent.

The glucoside is readily hydrolyzed by dilute acid and yields paranitrophenol and tetracetyl glucose. It is slowly hydrolyzed by dilute alkali in the cold. It is insoluble in water, fairly soluble in hot alcohol. It is readily soluble in xylene.

5. *p-Nitrophenol β-glucoside:* 20 gms. of tetracetylnitrophenol β-glucoside were shaken with 60 gms. of recrystallized barium hydroxide, dissolved in 1200 cc. of water, for 24 hours at 4°C. At the end of this time the mixture was nearly

clear. A small amount of unchanged glucoside was filtered off. The filtrate, containing barium acetate and p-nitrophenol β-glucoside was treated with an excess of washed CO_2. The barium carbonate was centrifuged off. The supernatant liquid was concentrated to complete dryness *in vacuo*. The yellow powder was scraped from the flask and dried for two days in a high vacuum over P_2O_5. It was then extracted with 300 cc. of hot absolute alcohol and filtered from the barium acetate by using a hot water funnel. The filtrate was cooled and allowed to stand in the ice box for 2 days. The crystals formed were filtered off and were recrystallized from absolute alcohol. About 8 gms. of p-nitrophenol β-glucoside were obtained as a glistening white product, soluble in alcohol, and, to some extent, in water. This compound was readily hydrolyzed by dilute mineral acid, yielding the reducing sugar and paranitrophenol. It is slowly hydrolyzed by dilute alkali in the cold.

The substance melted at 165°C. $[\alpha]_D^{25°} = \frac{-1.55 \times 100}{2 \times 0.974} = -79.6°$ (in methyl alcohol).

Analysis: 4.305 mgs. substance: 7.555 mgs. CO_2 and 2.030 mgs. H_2O.

$C_{12}H_{15}O_8N$.	Calculated:	C 47.83 per cent	H 5.02 per cent.
Found:		C 47.85 per cent	H 5.27 per cent.

6. *p-Aminophenol β-glucoside.* 5 gms. of p-nitrophenol glucoside were dissolved in 500 cc. of warm 95 per cent redistilled alcohol. To the mixture was added 0.2 gm. of platinum oxide catalyst (13). The substance was hydrogenated under atmospheric pressure in a hydrogenating apparatus. The theoretical amount of hydrogen gas, when corrected for temperature, pressure and vapor tension, was utilized in the reduction of the nitro to the amino glucoside. The reduction took place readily and quickly, and was completed within 30 minutes. The alcohol solution of p-aminophenol β-glucoside was filtered through a quantitative filter paper, and the filtrate carefully evaporated *in vacuo* to dryness. 50 cc. of absolute alcohol were added and the mixture was again evaporated to dryness. The glucoside was finally dissolved in 100 cc. of absolute alcohol and the flask was placed on ice. After 48 hours, the snow-white crystals of p-aminophenol β-glucoside were filtered from the clear mother liquors. 4.0 gms. were recovered. The compound is readily soluble in water. It is soluble in 95 per cent alcohol, though more difficultly soluble in absolute alcohol. It is readily hydrolyzed by dilute mineral acid, yielding the reducing sugar. It is also hydrolyzed by alkali in the cold. If the hydrochloride is treated with nitrous acid and the reaction mixture is poured into an alkaline solution of α-naphthol, a brilliant red dye is produced which precipitates on acidification.

The compound melts at 160–161°C. $[\alpha]_D^{26°} = \frac{-1.11 \times 100}{2 \times 0.867} = -64.1°$ (in methyl alcohol).

Analysis: 5.254 mgs. substance: 10.25 mgs. CO_2 and 2.925 mgs. CO_2.
$C_{12}H_{17}O_6N$. Calculated: C 53.12 per cent, H 6.32 per cent.
Found: C 53.05 per cent, H 6.23 per cent.

7. *Tetracetyl p-nitrophenol β-galactoside.* This compound was prepared exactly as was the corresponding glucoside. The yields were practically the same. It crystallizes from absolute alcohol as long glistening very pale yellow needles melting at 144–145°C.

$$[\alpha]_D^{24^\circ} = \frac{-0.17 \times 100}{2 \times 1.022} = -8.3^\circ \text{ (in chloroform)}$$

Analysis: 4.000 mgs. substance: 7.475 mgs. CO_2 and 1.685 mgs. H_2O.
$C_{20}H_{23}O_{12}N$. Calculated: C 51.16 per cent, H 4.94 per cent
Found C 50.96 per cent, H 4.71 per cent

8. *p-Nitrophenol β-galactoside.* This derivative was prepared exactly as was the glucoside. 25.4 gms. of p-nitrophenol tetracetyl β-galactoside yielded 11.9 gms. of p-nitrophenol β-galactoside. The compound crystallizes from absolute alcohol as colorless needles melting at 181–182°C.

Analysis: 4.100 mgs. substance: 7.175 mgs. CO_2 and 1.825 mgs. H_2O
$C_{12}H_{15}O_8N$. Calculated: C 47.83 per cent, H 5.02 per cent.
Found C 47.72 per cent, H 4.99 per cent.

9. *p-Aminophenol β-galactoside.* The reduction of p-nitrophenol β-galactoside was carried out in the same way as was that of the corresponding glucoside. The reduction does not seem to take place quite as rapidly as does that of the glucoside. The yields are practically quantitative. The compound was isolated from absolute alcohol as a white crystalline product soluble in water. It suffers hydrolysis when boiled with dilute mineral acid. When hydrolyzed solutions of the amino glucoside are boiled with Fehling's solution, a powerful reduction takes place.

The substance melts at 158–159°C. $[\alpha]_D^{25^\circ} = \frac{-74 \times 100}{2 \times 0.914} = -40.5^\circ$ (in methyl alcohol).

Analysis: 5.350 mgs. substance: 10.370 mgs. CO_2 and 3.160 mgs. H_2O.
$C_{12}H_{17}O_6N$. Calculated: C 53.12 per cent, H 6.32 per cent.
Found: C 52.85 per cent, H 6.61 per cent.

10. *Preparation of Serum Globulin.* 1 liter of normal horse serum was poured into 15 liters of distilled water. Dilute acetic acid was added until the maximum turbidity occurred. The mixture was placed in the ice chest for 48 hours. The supernatant liquid was then syphoned off and discarded. The precipitate was centrifuged in the cold. The globulin thus obtained was dissolved in physiological

salt solution and reprecipitated. A final globulin solution of 2 per cent concentration was employed for coupling with the glucosides.

11. *Preparation of Protein-diazophenol glucoside and galactoside.* 100 cc. of 2 per cent globulin solution were placed in a flask and cooled to 0°C. 100 cc. of N/50 NaOH, in physiological salt solution, were also cooled to 0°C. Then 1.00 gm. of p-aminophenol β-glucoside was dissolved in 25 cc. of water. 2 mols of N/1 hydrochloric acid were added. The mixture was cooled to 0°C. The theoretical quantity of sodium nitrite, dissolved in 10 cc. of water, was slowly added. After diazotization was complete and the solution showed no excess of nitrite, it was slowly added to the globulin, which had previously been mixed with the chilled alkali. A yellow color immediately developed which soon deepened to a dark wine-red. After standing for 30 minutes at 0° the mixture was treated with 50 cc. of chilled N/50 HCl in physiological salt solution. A precipitate of highly colored insoluble protein flocculated from the mixture. This protein was centrifuged from the colored supernatant liquid. The precipitate was then thoroughly extracted five times with a total of one liter of 3 per cent salt solution. The final washing was nearly colorless and protein free. The precipitate of protein diazophenol glucoside was suspended in 100 cc. of physiological salt solution and N/20 sodium hydroxide was cautiously added with stirring, until the suspension was completely in solution. The total dry weight of material was determined by evaporation, after dialysis, *in vacuo* to constant weight. The optical rotation was determined in a one-half decimeter tube using the wave length 760.8 mm. which has the same color as the azo-protein. The galactose diazophenol protein was prepared in exactly the same way as was the glucose derivative. It was finally taken up in 200 cc. of physiological salt solution.

Properties of the synthetic sugar proteins

Optical Rotation: A solution of the diazophenol glucoside protein, containing 9.725 mgs. of ash-free solid per cc., rotated the plane of polarized light −0.21° in a 0.5 decimeter tube.

$$[\alpha]^{25°}_{760.8\,\mu\mu} = \frac{-\ 0.21 \times 100}{0.5 \times 0.9725} = -\ 43.2°$$

A solution of the diazophenol galactoside protein, containing 5.200 mgs. of ash-free substance per cc., gave an observed rotation of −0.08° in a 0.5 decimeter tube.

$$[\alpha]^{25°}_{760.8\,\mu\mu} = \frac{-\ .08 \times 100}{0.5 \times 0.5200} = -\ 30.7°$$

Owing to the difficulty of securing a bright field of vision through these deeply colored solutions, the observed rotations probably suffered from an appreciable error. Six successive readings checked within 0.03°.

Reducing Sugars on Hydrolysis:—2 cc. of the protein solutions of the above concentration were placed in glass tubes together with 2 cc. of N/1 HCl. The tubes were sealed and placed in a boiling water bath. At the end of 3 hours and 5 hours respectively the tubes were removed, cooled, and centrifuged. A sugar analysis was made on 2 cc. of the hydrolysate by the method of Van Slyke and Hawkins (14). The galactose protein yielded approximately 10 per cent of reducing sugars, calculated as glucose. The glucose-protein yielded approximately 17 per cent of reducing sugars calculated as glucose.[2] There was no difference in the reducing value of the samples hydrolyzed for 3 hours when compared with those hydrolyzed for 5 hours.

The two synthetic sugar proteins appear to be different compounds. Animals have been immunized with these synthetic sugar proteins. The immunological results are reported in the following communication.

SUMMARY

1. The synthesis of p-aminophenol β-glucoside and p-aminophenol β-galactoside has been described.

2. These hexosides have been coupled to serum globulin. Two protein sugar complexes with different optical properties have been obtained.

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CHEMO-IMMUNOLOGICAL STUDIES ON CONJUGATED CARBOHYDRATE-PROTEINS

II. Immunological Specificity of Synthetic Sugar-Protein Antigens

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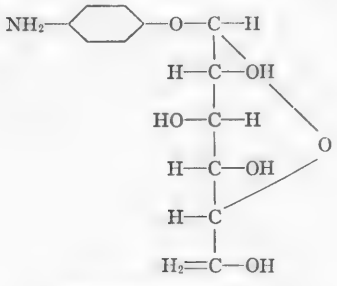
The function of carbohydrates as determinative substances in bacterial specificity is well illustrated in the immunological differentiation of the specific types of pneumococci and of Friedländer bacilli. Previous studies have demonstrated that the presence of type-specific polysaccharides in these encapsulated organisms determines the antigenic properties of the cell and the serological relationships of different strains.

In the course of investigations on the chemo-immunological nature of these complex bacterial antigens, the facts thus far ascertained lend support to the view that the specific polysaccharides function as true antigens only when combined with some other constituent of the cell. While the character of the substance which enters into combination with these complex sugars, and the nature of the linkage between them are still undetermined, it seems probable that the type-specific antigen consists of a protein or protein derivative conjugated with the specific carbohydrate; and that it is the latter component which orientates the specificity of the complex antigen thus formed. The possibility also exists that the polysaccharides which by themselves are non-antigenic, may, by reason of their acidic properties acquire antigenicity by combining to form salts with some basic constituent of the bacterial cell. This latter possibility, as well as the supposition that a mere change in the physical state of the polysaccharide as it exists in the intact cell may account for its antigenic behavior, seem in the light of present knowledge, to be

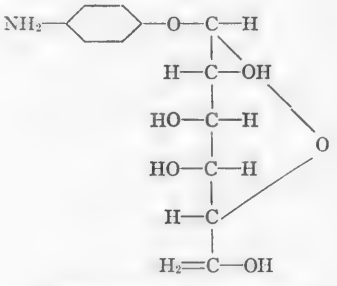
less likely than does the former view that the type-specific antigen consists of a conjugated carbohydrate-protein complex in which the carbohydrate radical determines the specificity of the whole.

With a view to the possible elucidation of the problems just stated, it appeared of considerable interest to determine the effect produced on the antigenic specificity of a protein by combining it with a relatively simple carbohydrate. This combination was effected, as described in the preceding paper (1), by synthesis of the p-aminophenol glucosides of glucose and galactose, and by coupling these diazotized glucosides with different proteins.

The experiment was made all the more exacting by the purposeful choice of two monosaccharides which have the same chemical formula and which differ from each other only in specific rotation and molecular configuration:—the groups on the fourth carbon atom in galactose forming the mirror image of the fourth carbon groups in glucose. The remainder of the molecule is the same in both sugars. The relationship of the p-aminophenol β-glucoside to p-aminophenol β-galactoside may be seen from the two structural formulae—



p-aminophenol β-glucoside



p-aminophenol β-galactoside

The diazonium derivatives of these two glucosides were attached to proteins by means of the linkage $-N=N-\langle\bigcirc\rangle-$ and the two synthetic sugar proteins thus derived were used as antigens.

The specificity of these conjugated carbohydrate-proteins was further tested by linking the same diazotized glucoside to two chemically distinct proteins derived from widely remote biological species.

It will be shown that immune sera prepared with these complex

antigens contain two separate kinds of antibodies; one variety stimulated by the conjugated sugar-protein, and the other evoked by the protein itself, varying amounts of which coexist unbound in the same solution. The immunological specificity of the synthetic sugar-proteins described in the present paper will be discussed, therefore, with reference to these two kinds of antibodies; 1. the anticarbohydrate antibodies (anti-S) and 2. the antiprotein antibodies (anti-P.). It will be shown further that each variety of antibody is specifically related to the corresponding component of the antigen; that the anti-protein antibodies exhibit the species specificity of the original protein, and that the antibodies reactive with the conjugated sugar-proteins are specific for unrelated proteins containing the same diazotized carbohydrate group.

The results of cross precipitin reactions, precipitin absorption and inhibition tests with immune sera prepared with the synthetic sugar-proteins are presented in the following protocols.

EXPERIMENTAL

Methods

Rabbits were immunized by the intravenous injection of solutions of the conjugated carbohydrate-protein antigens. The animals of one series received the antigen composed of purified globulin from horse serum coupled to diazo phenol glucoside, and those of another series were treated with the same protein combined with the diazo phenol galactoside. The rabbits of both series were injected with 2 cc. of the respective antigen daily for six doses and the course of injections was repeated at weekly intervals until a total of 36 cc. of antigen was given. Eight days after the last injection the rabbits were bled and the serum tested for precipitins against the homologous and heterologous antigens.

The antigenic material was prepared by the method described in the preceding paper, and was sterilized by passage through a Berkefeld filter. The same stock solution was used throughout the course of immunization. None of the rabbits showed any evidence of toxic symptoms following repeated injections.

Preparation of proteins:—The two proteins to which the diazo phenol glucosides were linked were serum globulin (horse) and crystalline egg albumin. The globulin was prepared from horse serum by precipitation with dilute acetic acid as described in the preceding paper. The crystalline egg albumin was made from native egg white by the method of Soerensen (2).

Preparation of carbohydrate-proteins:—By the methods described in the preceding paper (1) the following synthetic sugar-proteins were prepared:

1. phenol β glucoside-azo-globulin.
2. phenol β galactoside-azo-globulin.
3. phenol β glucoside-azo-albumin.
4. phenol β galactoside-azo-albumin.

For the sake of convenience the above preparations will be referred to respectively as gluco-globulin, galacto-globulin, gluco-albumin, and galacto-albumin.

Precipitin reactions:—The immune sera were in all instances used in constant amounts of 0.2 cc. A dilution of serum in the proportion of 2 parts of serum to 3 parts of salt solution was prepared and 0.5 cc. of this dilution, containing 0.2 cc. of the original serum, was added to 0.5 cc. amounts of the varying dilutions of the antigens as shown in the protocols.

Standardization of antigens:—For purposes of comparison, the sugar protein antigens were standardized on the basis of nitrogen-content; this method, however, does not indicate the amount of bound carbohydrate and hence is not a measure of the effective antigen complex.

I. ANTICARBOHYDRATE ANTIBODIES: (ANTI-S)

1. Precipitin reactions with Gluco-globulin Anti-Serum

Serum prepared by immunization with gluco-globulin was tested for the presence of precipitins against the homologous antigen and two other sugar-proteins, gluco-albumin and galacto-albumin. Both of the latter test substances contain egg albumin, a protein foreign to the immunizing antigen. In the case of gluco-albumin, however, the carbohydrate radical is the same as that present in the sugar-protein used for immunization, while in the case of galacto-albumin both the protein fraction and the sugar derivative are heterologous with respect to the original antigen. The results of cross precipitin tests are given in Table I.

The data presented in Table I show that gluco-globulin anti-serum reacts not only with gluco-globulin but also with gluco-albumin. The fact that antibodies stimulated by gluco-globulin are specifically reactive with gluco-albumin, which contains the homologous glucose derivative bound to a protein unrelated to that present in the immunizing antigen, demonstrates that the carbohydrate radical and not the protein molecule orientates the specificity of these conjugated sugar-proteins. The specificity of the orienting carbohydrate is further emphasized by the fact that antibodies reactive with gluco-albumin show no reaction with galacto-albumin in which an isomeric carbo-

hydrate is conjugated with the same protein. This is further proof that the chemical constitution of the sugar radical, regardless of the nature of the protein to which it is attached, determines the serological specificity of the conjugated antigen.

TABLE I

Precipitin Reactions of Gluco-Globulin Antiserum

Showing the specificity of sugar-proteins when the same carbohydrate derivative is combined with two serologically distinct proteins.

Anti-Gluco-Globulin Serum

Dilutions of antigens†	Carbohydrate-Protein Antigens		
	Gluco-globulin (horse)	Gluco-Albumin (egg)	Galacto-Albumin (egg)
1:1000	++±	++	−
1:5000	++++	++++	−
1:10,000	++++	++++	−
1:20,000	+++±	+++	−
1:40,000	+++	+	−
1:80,000	+	±	−
1:100,000	±	−	−

++++ = Complete precipitation with clear supernatant and formation of a compact, disc-like precipitate not easily disrupted by shaking—characteristic of type specific polysaccharide reactions with anti-pneumonococcus serum.

± = Faint turbidity.

− = No reaction.

† The antigens were standardized on the basis of nitrogen content. This method does not measure the amount of glucoside bound to protein.

2. *Precipitin reactions with Galacto-Globulin Anti-serum*

The cross precipitin reactions with galacto-globulin antiserum and three synthetic sugar-proteins, galacto-globulin, galacto-albumin and gluco-albumin, are given in Table II.

Specific precipitins in the serum of rabbits immunized with galacto-globulin react with solutions of galacto-globulin and galacto-albumin in approximately equal titre. These antibodies, however, fail to precipitate gluco-albumin. (Table II.) The specificity of the orienting sugar radical is again revealed in the cross precipitin reac-

tions between galacto-globulin and galacto-albumin. The carbohydrate derivative alone is common to both of these compounds, while the protein fraction of each is wholly dissimilar. The specific relationships between these sugar-proteins, therefore, appear to depend upon the nature of the particular carbohydrate component rather than upon the kind of protein to which it is linked.

TABLE II

Precipitin Reactions of Galacto-Globulin Antiserum

Showing the specificity of anti-S antibodies by cross-reactions with two serologically distinct proteins containing the same sugar-radical.

Anti-Galacto-Globulin Serum

Dilution of Antigen	Antigens		
	Galacto-Globulin	Galacto-Egg-Albumin	Gluco-Egg-Albumin
1:5000	++++	+++±	−
1:10,000	++++	+++±	−
1:20,000	+++	+++	−
1:40,000	++±	++	−
1:80,000	++	+	−
1:100,000	+	±	−

++++ = Complete precipitation with compact, disc-like precipitate.
− = No reaction.

The data presented in Table I and II may be summarized briefly as follows:—

1. When two chemically different carbohydrate derivatives are bound to the same protein, the newly formed compounds are serologically distinct from one another. Simple differences in the molecular configuration of the two isomers, glucose and galactose, although confined to one single carbon atom in the molecule, suffice to orientate antigenic specificity when corresponding glucosides of these sugars are coupled to the same protein.

2. When the same carbohydrate radical is conjugated with two chemically and serologically distinct proteins, both of the sugar-proteins thus formed acquire a common serological specificity.

3. The newly acquired specificity of these sugar-proteins is determined by the chemical constitution of the carbohydrate attached to the protein molecule.

3. Specific Inhibition of Precipitin Reactions by Homologous Glucosides

In his studies on complex antigens Landsteiner (3) has shown that immune sera prepared with azo-proteins are markedly specific, precipitating unrelated proteins which contain the same azo groups. However, the simple azo compounds by themselves are not antigenic and are not precipitable in azo-protein antiserum. Nevertheless, when they are added to the immune serum prepared with protein coupled to the same diazotized compounds they specifically inhibit the antibodies from reacting subsequently with the homologous antigen.

Since in the present study the diazotized phenol glucosides of glucose and galactose exhibit immunological properties analogous to those of the diazotized amino compounds described by Landsteiner, the inhibiting action of these carbohydrate substances on the precipitins of homologous immune sera was studied.

Inhibition Test: Solutions of p-aminophenol β-glucoside and p-aminophenol β-galactoside in concentrations of 0.1 M. and 0.01 M. were added in amounts of 0.2 and 0.1 cc. to a constant unit of immune serum (0.2 cc.) and made up to volume by addition of salt solution. The mixtures were incubated at 37°C. for 2 hours, and the tubes examined for the presence of precipitate. To the test mixtures, 0.5 cc. of sugar protein antigen containing the homologous carbohydrate derivative was then added in optimal dilution, and the tubes again incubated for 2 hours. Readings were made at the end of the period of incubation and after 24 hours in the ice-box.

The results of the inhibition tests with homologous glucosides are given in Tables III and IV.

Since the inhibiting action of the homologous glucosides on specific precipitins is similar in each instance, the results will be discussed together. (Tables III and IV.) It is apparent that the glucosides by themselves are not precipitated in the presence of immune sera prepared with protein containing the homologous diazotized compounds.

It is also evident that although the carbohydrate substances alone fail to cause precipitation in immune serum, they, nevertheless, specifically inhibit the precipitating antibodies from reacting with the homologous sugar-protein when the latter is subsequently added to the serum mixture. The specificity of the reaction is shown by the fact that while the addition to homologous serum of 0.1 cc. of 0.01 M solution of the corresponding glucoside completely inhibits precipitation

when the specific carbohydrate-protein is added later, the addition of a ten to twenty-fold concentration of a heterologous glucoside, under the same conditions, exerts no inhibition on the precipitating antibodies.

TABLE III

Specific Inhibition by Homologous Glucoside of Precipitin Reactions with Gluco-Globulin Serum

	Gluco-Globulin Serum	Glucoside		Galactoside		Salt solution to volume	Incubation—2 hrs. at 37°C. No precipitate formed.	Antigen	Result	
		0.1 M.	0.01 M.	0.1 M.	0.01 M.			Gluco-Egg Albumin 1:10,000	2 hrs. 37°C.	24 hrs. ice-box
	cc.	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*		*cc.*		
1	0.2	0.2	—	—	—	0.3		0.5	—	—
2	0.2	0.1	—	—	—	0.4		0.5	—	—
3	0.2	—	0.2	—	—	0.3		0.5	—	±
4	0.2	—	0.1	—	—	0.4		0.5	±	+
5	0.2	0.2	—	—	—	0.8		—	—	—
6	0.2	—	—	—	—	0.5		0.5	++	++++
7	0.2	—	—	0.2	—	0.3		0.5	++	++++
8	0.2	—	—	0.1	—	0.4		0.5	++	++++
9	0.2	—	—	—	0.2	0.3		0.5	++	++++
10	0.2	—	—	—	0.1	0.4		0.5	++	++++
11	0.2	—	—	0.2	—	0.8		—	—	—
12	0.2	—	—	—	—	0.7		—	—	—
13	—	—	—	—	—	0.7		0.5	—	—
14	—	0.2	—	—	—	1.0		—	—	—
15	—	—	—	0.2	—	1.0		—	—	—

+++ = Complete precipitation.
— = No reaction.

The simple monosaccharides,—glucose and galactose—from which the respective glucosides were synthesized, have been found to be inert in specific precipitin and inhibition reactions with immune sera prepared with protein united to the corresponding diazotized glucosides.

The data presented in Tables III and IV, may be summarized as follows:—

1. The unconjugated glucosides, although themselves not precipitable in immune serum, inhibit the reaction between the homologous sugar-protein and specific antibody.

2. This inhibition is specific, since heterologous glucosides exert no inhibiting action.

TABLE IV

Specific Inhibition by Homologous Galactoside of Precipitin Reactions with Galacto-Globulin Serum

	Galacto-Globulin Serum	Galactoside		Glucoside		Salt solution to volume	Incubation—2 hrs. 37°C. No precipitate formed.	Antigen	Result	
		0.1 M.	0.01 M.	0.1 M.	0.01 M.			Galacto-Egg Albumin 1:10,000	2 hrs. 37°C.	24 hrs. ice-box
	cc.	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*		*cc.*		
1	0.2	0.2	—	—	—	0.3		0.5	—	—
2	0.2	0.1	—	—	—	0.4		0.5	—	—
3	0.2	—	0.2	—	—	0.3		0.5	—	±
4	0.2	—	0.1	—	—	0.4		0.5	±	±
5	0.2	0.2	—	—	—	0.8		—	—	—
6	0.2	—	—	—	—	0.5		0.5	++	+++
7	0.2	—	—	0.2	—	0.3		0.5	++	+++
8	0.2	—	—	0.1	—	0.4		0.5	++	+++
9	0.2	—	—	—	0.2	0.3		0.5	++	+++
10	0.2	—	—	—	0.1	0.4		0.5	++	+++
11	0.2	—	—	0.2	—	0.8		—	—	—
12	0.2	—	—	—	—	0.7		—	—	—
13	—	—	—	—	—	0.7		0.5	—	—
14	—	0.2	—	—	—	1.0		—	—	—
15	—	—	0.2	—	—	1.0		—	—	—

3. These glucosides exhibit the properties of haptens; they are non-antigenic substances which are specifically reactive, as shown by inhibition tests, with antibodies induced by proteins containing the homologous diazotized compounds.

II. ANTIPROTEIN ANTIBODIES (ANTI-P)

In the preceding experiments the immune sera prepared with conjugated sugar-proteins have been analyzed only with reference to

the presence of antibodies specifically related to the particular carbohydrate radical introduced into the protein molecule. These so-called anti-carbohydrate antibodies (Anti-S) are specifically reactive with unrelated proteins containing the same diazotized glucosides. However, in addition to the antibodies just described, the sera also contain

TABLE V

Precipitin Reactions of Gluco-Globulin Antiserum

Showing the species specificity of the antiprotein antibodies in cross reactions with antigens containing the same protein molecule.

Gluco-Globulin Serum

Dilution of Antigens	Carbohydrate-protein Antigens					
	Gluco-Globulin (horse)	Galacto-Globulin (horse)	Globulin (horse)	Gluco-Albumin (egg)	Galacto-Albumin (egg)	Albumin (egg)
1:5000	$\left(\frac{++++}{xxxx}\right)$	xxxx	xxxx	+++±	–	–
1:10,000	$\left(\frac{++++}{xxxx}\right)$	xxx	xxxx	+++	–	–
1:20,000	$\left(\frac{+++}{xxx}\right)$	xx	xx	++±	–	–
1:40,000	$\left(\frac{+++}{xxx}\right)$	x	$\frac{xx}{-}$	+±	–	–
1:80,000	$\left(\frac{+}{x}\right)$	$\frac{x}{-}$	x	±	–	–
1:100,000	$\left(\frac{±}{x}\right)$	–	$\frac{x}{-}$	–	–	–

x = Reactions with common species protein by Anti-P antibodies.
\+ = Reactions with specific carbohydrate group by Anti-S antibodies.

anti-protein antibodies which are reactive only with the particular kind of protein present in the immunizing antigen. The following experiments deal with the occurrence, the species specificity and specific absorption of the anti-protein (Anti-P) antibodies.

1. Occurrence: In the conjugated antigens used for immunization

the total amount of protein present was estimated by determining the nitrogen content of the solution. However, this method of standardization is obviously inadequate, since it provides no measure of the relative amount of protein bound by the diazotized glucoside. As an excess of protein was always used for coupling with the carbohydrate, a greater or less amount of unbound protein remained free in solution depending upon the variable factors involved in the chemical reactions

TABLE VI

Precipitin Reactions of Galacto-Globulin Antiserum

Dilution of Antigen	Galacto-Globulin	Gluco-Globulin	Globulin	Gluco-Albumin	Galacto-Albumin	Albumin
1:5000	$\left(\frac{++++}{x}\right)$†	$\frac{x}{-}$	$\frac{x}{-}$	−	++++‡	−
1:10,000	$\left(\frac{++++}{x}\right)$	$\frac{x}{-}$	$\frac{x}{-}$	−	++++	−
1:20,000	$\left(\frac{+++}{x}\right)$	x	−	−	++±	−
1:40,000	$\left(\frac{++}{x}\right)$	$\frac{x}{-}$	−	−	++	−
1:80,000	$\left(\frac{+}{x}\right)$	−	−	−	+	−
1:100,000	$\left(\frac{x}{-}\right)$	−	−		−	

† = Bulky precipitate representing combined action of Anti-S and Anti-P.
‡ = Compact disk-like precipitate characteristic of Anti-S reactions.

associated with the process of diazotization. The occurrence of antiprotein antibodies in the serum of rabbits immunized with gluco-globulin and galacto-globulin is, therefore, presumably due to the presence in both antigens of an excess of common protein free in solution.

The differences observed in the titre of antiprotein precipitins in the two sera, (Tables V and VI) appear to be attributable to corresponding differences in the relative amount of free protein in each

antigen. This opinion is strengthened by the fact that the gluco-globulin antigen which yielded a high concentration of Anti-P precipitins was found on analysis to contain approximately 63 per cent more protein by weight than was present in the solution of galacto-globulin. This difference is reflected in the relative concentration of precipitins, since the serum prepared with the antigen having the greater amount of protein contains the higher concentration of anti-protein antibodies.

In this connection it is interesting to observe that, despite the differences in anti-protein response, both antigens gave rise to specific anticarbohydrate antibodies in approximately equal titre.

TABLE VII

Precipitin Reactions of Anti-horse Serum with Sugar-proteins containing horse serum Globulin

Anti-horse Serum

Dilution of Antigen	Gluco-Globulin	Galacto-Globulin	Original Globulin
1:1000	xx	xx	xxx
1:5000	xxxx	xxxx	xxxx
1:10,000	xxx	xxx	xxxx
1:20,000	xx	xx	xxx
1:40,000	x	x	xx
1:80,000	x	x	x

The antiprotein antibodies evoked by immunization with gluco-globulin and galacto-globulin, are presumably due to the presence in the antigens of free protein unbound by the diazotized glucosides. The supposition that, even in the absence of free protein, the sugar-protein compex alone may give rise to two qualitatively different antibodies, each specifically related to the corresponding constituent of the antigenic complex, affords an interesting alternative explanation of the occurrence of the two antibodies in the same immune serum. This concept involves the assumption that the binding of the diazo-glucoside to the protein does not entirely mask the groups essential to the specificity of the protein molecule, and that the carbohydrate radical through conjugation acquires specific antigenicity while the protein molecule retains in part its original antigenic properties.

2. *Specificity:* The antiprotein antibodies commonly found in these

immune sera exhibit only the species specificity of the particular kind of protein present. Since the same protein was used in preparing both test antigens, it is not surprising that a serum obtained with one shows cross precipitation with solutions of the other. The antiprotein precipitins in both sera react only in the presence of the homologous protein of the same species. They do not precipitate proteins unrelated to that present in the immunizing antigen. In this respect they are easily differentiated from the anticarbohydrate antibodies which, as shown in the present study, react with heterologous protein containing the same sugar radical.

TABLE VIII

Differentiation of Anti-S and Anti-P Antibodies by Specific Inhibition with Homologous Glucoside

Gluco-horse-Globulin Serum from rabbit*	Antigens							
	Gluco-Albumin (Egg)†				Globulin (Horse)‡			
	1:5000	1:10,000	1:20,000	1:40,000	1:5000	1:10,000	1:20,000	1:40,000
With the addition of Glucoside	−	−	−	−	xx	xx	xx	x
Without the addition of Glucoside	+++±	++++	+++	+±	xxx	xx	xx	x

* Precipitating serum prepared by immunization of rabbit with solution of glucoside coupled to globulin obtained from horse serum.

† Demonstrating anti-carbohydrate (anti-S) reactions.

‡ Demonstrating anti-protein (anti-P) reactions.

The species specificity of the antiprotein precipitins is demonstrated in Tables V and VI. The species relationship between homologous protein and its antibody is again illustrated in Table VII, in which it is evident that a precipitating serum prepared by immunization of rabbits with plain horse serum reacts with solutions of gluco- and galacto-globulin containing in common a native protein of the same animal origin. These protein-antiprotein reactions conform in all respects to the well known principles of species specificity. They are included here only to emphasize by contrast the preceding observations on the new specificity acquired by proteins of unrelated species when a simple sugar radical is attached to the protein molecule.

3. Specific Absorption: By fractional absorption with globulin alone, the antiprotein precipitins may be removed from an immune serum without appreciably diminishing the titre of the specific anti-carbohydrate antibodies. Thus, a distinct qualitative difference between these two antibodies had been demonstrated by specific absorption tests.

The specific inhibition of the anticarbohydrate antibody by the homologous glucoside has already been shown in Tables III and IV. This same technique has been employed in the differentiation of anti-S and anti-P. The anticarbohydrate antibodies contained in a serum prepared with gluco-globulin may be specifically inhibited by the addition of the homologous glucoside without impairing the titre of the antiprotein antibodies present in the same serum. This method is simpler than the absorption technique and is particularly applicable when dealing with antigens in the soluble form.

To summarize then, it may be stated that the occurrence of anti-protein precipitins in sera prepared with sugar-proteins appears to be related to the presence of free proteins in the solution. There is no evidence at present suggesting that this antibody arises as the result of dissociation of the sugar-protein complex either *in vitro* or *in vivo*. The antiprotein antibody exhibits the protein specificity of the species from which it is derived. It can be removed from immune sera by specific absorption, without loss in the titre of the coexisting anti-carbohydrate antibodies. Conversely, the anticarbohydrate anti-bodies can be specifically inhibited from reacting by the addition of the homologous glucoside without diminishing the activity of the antiprotein precipitins present in the same serum.

DISCUSSION

Obermeyer and Pick (4) studied the changes in specificity brought about by subjecting proteins to various chemical reactions, such as that brought about by iodine or nitric acid. They found that the chemical changes thus induced gave rise to new antigens with altered serological properties. Precipitins stimulated by the modified protein were specifically reactive with unrelated proteins similarly treated. The chemically altered proteins lost their species specificity but acquired a new specificity common to all proteins which had under-

gone the same chemical treatment as that to which the immunizing antigen had been subjected.

Landsteiner first prepared complex antigens, the chemo-specific groups of which consisted of known chemical substances attached to the protein molecule. The important and unexpected results of the investigations gave rise to the new concept that complex antigens may contain specifically reacting substances which by themselves are not antigenic but which acquire antigenicity when they are united to protein. Furthermore, Landsteiner showed that antibodies induced by these artificially prepared antigens react with the simple chemical compounds unattached to the antigenic protein. These specifically reactive but non-antigenic substances he called haptens,—substances which independently possess the specific antibody binding property, but lack the immunizing power of the whole antigen of which they form a part.

Complex antigens containing non-protein fractions are now known to occur in nature; for example, 1) the Forssman antigen of animal cells, the specificity of which is oriented by the presence of substances presumably lipoidal, and 2) the complex antigens of certain bacterial cells, in which the presence of specific polysaccharides determines antigenic specificity.

The significance of carbohydrates in the antigenic structure of bacteria lends added interest to the results of the present study in which it has been found that simple sugars have a determinative influence on the specificity of the proteins to which they have been chemically bound. As in the case of the type-specific antigen of Pneumococcus, the serological properties of these artificially prepared sugar-proteins appear to be determined by the chemical structure of the carbohydrate radical rather than by the character of the protein to which it is attached. So specific are these relationships that a serum prepared by immunization with gluco-globulin, the protein of which is derived from horse serum, reacts specifically with egg albumen to which the same sugar derivative has been coupled. Proteins of widely remote biological origin, therefore, when combined with the same carbohydrate radical have been shown to acquire in common a new and reciprocal specificity. Similarly, it has been found that when the same protein is combined separately with the corresponding

derivatives of two different sugars the resulting compounds are, in each instance, serologically distinct and specific.

In addition, the present work reveals the important fact that derivations of the two simple monosaccharides—glucose and galactose—which differ one from the other only in the spatial configuration of a single carbon atom, exhibit distinct immunological specificity when combined with the same or different proteins. Thus, for the first time, it has been shown by direct experimental evidence that asymmetry of the carbon atoms in the sugar radical alone suffices to determine differences in the specificity of sugar-protein antigens. Furthermore, these simple carbohydrate substances mask the species specificity of the protein molecule to which they are attached, and confer upon the combined antigen a new specificity which is dependent solely upon the chemical structure of the simple sugar radical.

The particular hexosides used in the present study were synthesized from glucose and galactose in form of the corresponding p-aminophenol β-glucoside and galactoside. These substances, when injected into the animal body, apparently do not stimulate the formation of demonstrable antibodies in the blood serum of the treated rabbits. At least it may be stated that attempts to induce antibody formation have failed despite the fact that both glucosides contain approximately five per cent of nitrogen. This lack of ability to stimulate the formation of antibodies is interesting in the light of Ford's original observation (5) that a nitrogenous glucoside isolated from a variety of poisonous mushroom produced antibodies which neutralized the hemolytic action of the glucoside. It may be mentioned in passing that the glucosides used in the present study are not hemolytic for rabbit blood cells. Moreover, both glucosides fail to cause the formation of a precipitate when added to an immune serum prepared with the homologous sugar-protein. The lack of specific precipitation in immune serum may be referable to the fact that the simple sugar derivatives are crystalloids and of relatively small molecular size when compared with the colloidal and highly complex sugars of Pneumococcus which react so readily in precipitin tests with specific antibacterial sera. However, despite the lack of specific precipitability, the homologous glucoside when added to the test mixture inhibits the precipitin reaction between the corresponding sugar-protein and its specific antibody.

The mechanism of the inhibition is not as yet clear. It is definite, however, that the inhibitory action of the sugar radical is specific. The precipitins for galacto-protein compounds, for example, are inhibited only in the presence of the homologous galactoside and are not affected by the addition of the heterologous glucoside (Tables III and IV). Moreover, the inhibition reaction, as Landsteiner has pointed out in the case of the azo-proteins, affords a specific method for the serological differentiation of simple chemical substances,—in this instance isomeric sugar derivatives—which by themselves are not antigenic and non-precipitable in immune sera. By reason of their serological specificity it appears justifiable to place these artificially prepared glucosides in the class of carbohydrate haptens, the most conspicuous examples of which are the specific polysaccharides naturally found in certain micro-organisms.

The results obtained with these synthetic sugar-proteins offer suggestive lines of approach to a further study of the chemo-immunological nature of those complex bacterial antigens in which carbohydrates are known to be the specific substances.

SUMMARY

1. When two chemically different carbohydrate derivatives are bound to the same protein, the newly formed antigens exhibit distinct immunological specificity.

2. When the same carbohydrate radical is conjugated with two chemically different and serologically distinct proteins both of the sugar-proteins thus formed acquire a common serological specificity.

3. The newly acquired specificity of the artificially prepared sugar-proteins is determined by the chemical constitution of the carbohydrate radical attached to the protein molecules. Simple differences in the molecular configuration of the two isomers,—glucose and galactose—suffice to orientate protein specificity when the corresponding glucosides of the two sugars are coupled to the same protein.

4. The unconjugated glucosides, although themselves not precipitable in immune serum, inhibit the reaction between the homologous sugar-protein and its specific antibody. The inhibition test is specific.

5. The sugar derivatives unattached to protein exhibit the properties of carbohydrate haptens; they are non-antigenic but specifically

reactive, as shown by inhibition tests, with antibodies induced by proteins containing the homologous diazotized glucoside.

6. The specificity of artificially prepared sugar-proteins is discussed with reference to the chemo-immunological nature of the bacterial antigens containing complex sugars.

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